EFFECTS OF SPACE FLIGHT, CLINOROTATION, AND CENTRIFUGATION ON THE GROWTH AND METABOLISM OF *ESCHERICHIA coli*

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Effects of Space Flight, Clinorotation, and Centrifugation on the Growth and Metabolism of *Escherichia coli*

Thesis directed by Dr. Paul W. Todd and Dr. David M. Klaus

Previous experiments have shown that space flight stimulates bacterial growth and metabolism. An explanation for these results is proposed, which may eventually lead to improved terrestrial pharmaceutical production efficiency. It is hypothesized that inertial acceleration affects bacterial growth and metabolism by altering the transport phenomena in the cells' external fluid environment. It is believed that this occurs indirectly through changes in the sedimentation rate acting on the bacteria and buoyancy-driven convection acting on their excreted by-products.

Experiments over a broad range of accelerations consistently supported this theory. Experiments at 1 *g* indicated that higher concentrations of excreted by-products surrounding bacterial cells result in a shorter lag phase. Nineteen additional experiments simulated 0 *g* and 0.5 *g* using a clinostat, and achieved 50 *g*, 180 *g*, and 400 *g* using a centrifuge. These experiments showed that final cell density is inversely related to the level of acceleration. The experiments also consistently showed that acceleration affects the length of the lag phase in a non-monotonic, yet predictable, manner. Additional data indicated that *E. coli* metabolize glucose less efficiently at hypergravity, and more efficiently at hypogravity. A space-flight experiment was also performed. Samples on orbit had a statistically significant higher final cell density and more efficient metabolism than did ground controls. These results, which were similar

to simulations of 0 g using a clinostat, support the theory that gravity only affects bacterial growth and metabolism indirectly, through changes in the bacteria's fluid environment.

Evidence of buoyancy-driven convection associated with bacterial growth was also obtained by photographing a plume rising from metabolizing bacteria. These plumes were analyzed mathematically. Additional analysis, using a computer simulation, investigated the effects of diffusion and sedimentation. All of the analyses agreed with observed results and supported the proposed theory.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Many previous experiments have examined the effects of space flight on bacteria. With a few exceptions (Bouloc and D'Ari, 1991; Gasset *et al.*, 1994), a majority of findings indicate that reduced gravity stimulates the growth of suspended bacterial cultures. These experiments have consistently shown that space flight results in a shorter lag phase by approximately 4 to 8 hours (Klaus *et al.*, 1997; Mennigmann and Lange, 1986; Kacena *et al.*, 1997; Manko *et al.*, 1987) and almost twice as high a final cell density as comparable ground controls (Klaus *et al.*, 1997; Kordium *et al.*, 1980; Ciferri *et al.*, 1986; Mattoni, 1968; Mennigmann and Lange, 1986). Many other experiments have shown that antibiotics are less effective on orbit (Tixador *et al.*, 1981, 1985, 1994; Lapchine *et al.*, 1986, 1987, 1988, 1990; Klaus 1994). Two recent experiments also indicate that space flight stimulates the production of secondary metabolites (Lam *et al.*, 1998, 1999).

In addition to these investigations on orbit, a few bacterial experiments have been conducted on the ground using a centrifuge to achieve accelerations greater than 1 *g* or a clinostat to simulate some aspects of space flight. Experiments using a centrifuge have reported either no differences (Lapchine *et al.*, 1990; Bouloc and

D'Ari, 1991) or significantly less growth compared with 1 *g* controls (Montgomery *et al.*, 1963). Experiments on a clinostat have had similar results to those in space (Klaus *et al.*, 1998b; Mattoni, 1968; Mennigmann, 1994).

Many theories have been proposed to explain these findings. However, no one has identified a physical mechanism that can explain all of these results. A literature review also revealed that it is still unknown if the specific biomass yield (mass of new cells per mass of nutrient consumed) is affected by acceleration.

1.2 OBJECTIVES

The research in this dissertation addresses both of these issues. Bacterial growth and metabolism are investigated over a wide range of accelerations in an attempt to identify an underlying physical mechanism. This is accomplished to test the following primary hypothesis:

Primary Hypothesis:

Inertial acceleration affects the growth and metabolism of suspended E. coli cultures by altering the transport phenomena in the external fluid environment of the cells.

This main hypothesis is tested by five sub-hypotheses. These are shown on the following two pages in italics and are divided into three categories. The categories address how accelerations from 0 *g* to approximately 1,000 *g* are expected to affect the final cell density, the lag phase, and the metabolism of *E. coli*.

1. **Effect of Acceleration on Final Cell Density** (shown graphically in Figure 1-1)

   Final cell density is inversely related to the level of acceleration (including real and simulated hypogravity).

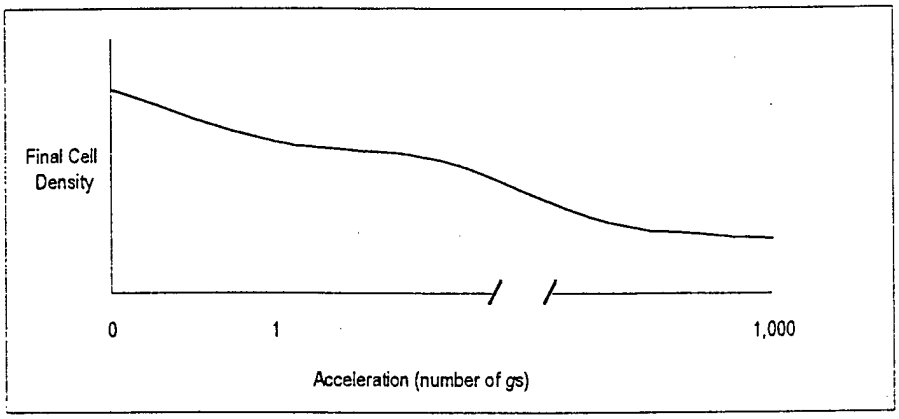

Figure 1-1 Hypothesized Effect of Acceleration on Final Cell Density

2. **Effect of Acceleration on the Lag Phase** (shown graphically in Figure 1-2)

   a. *For accelerations at and below 1 g (real and simulated), the length of the lag phase is directly related to the level of acceleration.*

   b. *Accelerations between approximately 10 g and 100 g result in a shorter lag phase than 1 g controls or experiments in hypogravity.*

   c. *As the acceleration increases from approximately 100 g to 1,000 g, the lag phase increases, eventually resulting in no difference relative to 1 g controls.*

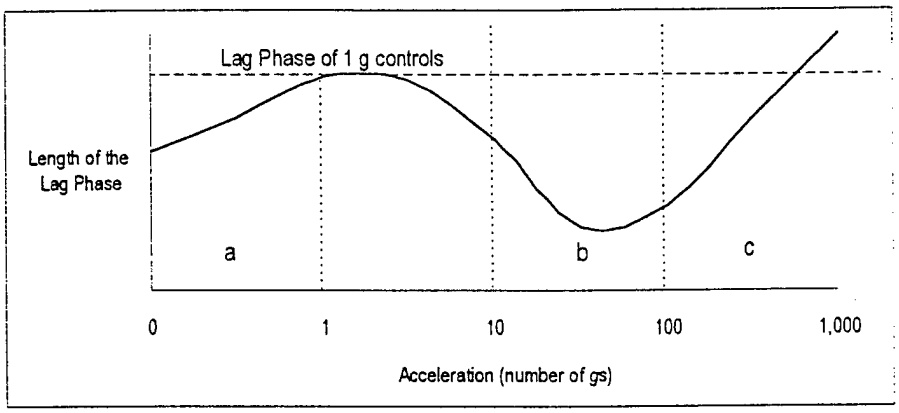

Figure 1-2 Hypothesized Effect of Acceleration on the Length of the Lag Phase. To include all three hypotheses about the lag phase on one plot, the abscissa is a log scale for accelerations greater than 1 *g*. The regions labeled a, b, and c correspond to the hypotheses listed above.

3. **Effect of Acceleration on Metabolism** (shown graphically in Figure 1-3)

The mass of new cells normalized per mass of glucose consumed is inversely related to acceleration (including real and simulated hypogravity).

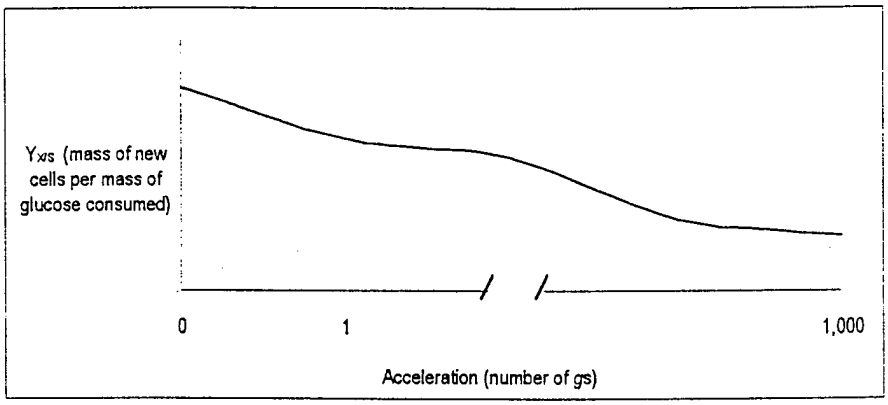

Figure 1-3 Hypothesized Effect of Acceleration on the Specific Biomass Yield

These hypotheses are derived from a new physical model proposed in this dissertation. This model claims inertial acceleration alters bacterial growth and metabolism through changes in the bacteria's sedimentation rate and buoyancy-driven convection acting on the excreted by-products. It is postulated that these two gravity-dependent forces alter the transport phenomena in the fluid environment surrounding bacteria. This is believed to affect the final cell density, the length of the lag phase, and the efficiency of metabolism by suspended bacterial cultures.

1.3 THESIS OUTLINE

Chapters 2 and 3 provide the necessary background information. Chapter 2 gives a comprehensive review of previous research on bacteria in modified inertial environments. This includes the effects of acceleration on bacterial growth, antibiotic

effectiveness, and bacterial metabolism. This chapter also discusses various explanations for the observed changes. Chapter 3 describes the general materials and methods used throughout this dissertation.

Chapter 4 examines an existing theory that claims acceleration primarily affects bacterial growth through changes in the cell's sedimentation rate. This theory is tested using ground experiments and computer modeling. Based upon these results, the chapter concludes by proposing a new physical model to explain how acceleration affects bacterial growth. It is believed that, in addition to sedimentation acting on the bacterial cells, buoyancy-driven convection also acts on the cells' excreted by-products. The absence of this force on orbit is believed to be the primary physical mechanism that can explain the reports of a shorter lag phase in space.

Based upon this proposed physical model, Chapter 5 outlines the four hypotheses stated above concerning the effects of acceleration on the final cell density and the lag phase. These hypotheses are tested with a total of 19 ground experiments, which support the proposed model.

Chapter 6 continues investigating the possibility of buoyancy-driven convection associated with bacterial growth. This includes a photograph of a plume rising from growing bacteria and mathematical analysis of the plume. Analysis shows the plume velocity is significantly greater than the sedimentation rate of *E. coli*. It also indicates this force can explain the non-linear relationships between acceleration and growth kinetics observed in Chapter 5.

Chapter 7 investigates the effects of acceleration on the metabolism of *E. coli*. Based upon the proposed physical model, it is believed that cell growth normalized per mass of glucose consumed is greater at lower levels of acceleration. This hypothesis is investigated using ground experiments as well as an experiment on orbit. Results support the hypothesis and proposed model.

Chapter 8 explores the economics of some potential industrial applications associated with this research. These include estimating the potential economic benefit of transferring knowledge gained from space-based research to terrestrial fermentation, as well as the potential for economically profitable space-based bioprocessing.

Chapter 9 summarizes the results of this dissertation and outlines some areas for further research.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter provides a comprehensive review of bacterial experiments subject to various inertial accelerations. The majority of these experiments have investigated the effects of inertial acceleration on bacterial growth. Other experiments have studied the effectiveness of antibiotics, the effects of space radiation, or bacterial metabolism at different levels of acceleration. The results from all of these experiments are presented along with various explanations for the observed changes.

2.2 VARYING INERTIAL ACCELERATION

There are many ways to vary the acceleration forces acting on a body. A centrifuge is generally used to simulate gravitational forces greater than 1 *g* (hypergravity). Although they can do this over a wide range of values, a centrifuge on Earth can not decrease the force of acceleration below 1 *g*. Gravitational forces less than 1 *g* (hypogravity) are achieved in a free-fall environment, and space flight is the only way to remain in hypogravity for more than a few minutes. Space flight does not achieve a true 0 *g* environment, though. Due to disturbances such as drag, gravity

gradients, and thruster firings, shuttle missions can only achieve about 10^{-5} g (Witherow, 1991).

Some aspects of space flight can also be simulated on the ground with the use of a clinostat. This device rotates a horizontal, fluid-filled tube at a constant rate with the axis of rotation perpendicular to gravity. If the rotational rate is sufficiently high, the displacement due to sedimentation is insignificant relative to Brownian motion. If this rotational rate is also slow enough to prevent significant centrifugal forces, a state of "functional weightlessness" can be achieved on the ground. At a rotational rate of approximately 2 to 3 RPM, a typical clinostat generates a maximum centrifugal force of about 10^{-4} g (Klaus *et al.*, 1998b). In this environment, bacterial cultures remain colloidal as they do on orbit.[1]

It is important to point out that the force of gravity still acts on cells in a clinostat, and the magnitude of this force does not change. However, the direction of gravity's vector constantly changes, which reduces the effects of convection and sedimentation. Therefore, a clinostat can only simulate the "indirect" effects of reduced gravity, which alter the fluid environment around the cells through sedimentation and buoyancy. The "direct" effects of gravity, causing such things as stress on the cellular membrane, are still present in samples on a clinostat.

[1] Section 3.2 discusses the equations governing sedimentation and diffusion on a clinostat, including a description of a new method used to simulate partial gravity with an inclined clinostat.

2.3 REVIEW OF BACTERIAL EXPERIMENTS IN SPACE

Many experiments involving microorganisms have been performed in space since the 1960s. Some of these experiments are summarized in a number of review articles (Alpatov *et al.*, 1990; Cioletti *et al.*, 1991; Dickson, 1991; Gmünder and Cogoli, 1988; Kern and Hock, 1993; McLaren, 1990; Mennigmann, 1994; Mesland, 1987; Taylor, 1974, 1977, 1986; Tixador *et al.*, 1981; Volkmann, 1988). While there is certainly controversy surrounding some findings, most research has shown that, "gravity influences the functioning of prokaryotic and eukaryotic cells" (Mesland, 1987).

This was quite unexpected before the spacelab era. Based upon theoretical research, many scientists did not expect to find any effect of microgravity on single cells smaller than about 10 μm (Babskiy, 1987; Kondo, 1968; Pollard, 1965). Despite these predictions, recent research has indicated that reduced gravity can affect microorganisms in a number of ways. Some processes are enhanced; others are inhibited; while others do not vary in microgravity (Volkmann, 1988).

There is even evidence to suggest that cells of all sizes and organizational complexity under certain circumstances can sense gravity, even if they are not specialized to do so (Gmünder and Cogoli, 1988). However, typical of the controversy surrounding the effects of microgravity, one researcher has recently presented data that indicated "individual one-celled organisms showed no signs of any gravity-dependent processes" (Parfenov, 1990). These different conclusions of the general effects of microgravity are typical of the more specific investigations presented

in this chapter. In some cases there are inconsistencies in the results and in the conclusions drawn from those results.

Table 2-1 provides a brief overview of specific results from bacterial experiments conducted in space. The experiments are divided into categories based upon their areas of investigation. Experiments involving bacterial growth have examined the length of the lag phase, the growth rate, and the final cell density on orbit (reference the three columns under "Bacterial Growth" in Table 2-1). Other experiments with bacteria in space have studied the effectiveness of antibiotics, the effects of space radiation, and the production of spores and secondary metabolites. Some of these results have been more repetitive than others and serve as a basis for identifying physical mechanisms behind the observed changes. For example, the column labeled "Antibiotic Effectiveness" in Table 2-1 indicates it is widely accepted that antibiotics are less effective in space. In contrast, findings in other categories have not been as consistent.

The following sections of this chapter will summarize the results of each of these categories, examining every column of Table 2-1 in detail. When applicable, results from the few experiments using a centrifuge or clinostat are also presented. At the conclusion of each section, the various theories behind the observed changes are discussed.

Table 2-1 Summary of Bacterial Experiments in Space

Reference	Bacterial Growth			Other Experiments			
	Length of Lag Phase	Growth Rate	Final Cell Density	Antibiotic Effectiveness	Effects of Radiation	Production of Spores and Metabolites	Miscellaneous
Zhukov-Verezhnikov, 1962					—		
Mattoni, 1968			H				L
Taylor and Zaloguev, 1978				L			
Kordium *et al.*, 1980			H				
Tixador *et al.*, 1981, 1985; Lapchine *et al.*, 1987				L			H
Ciferri *et al.*, 1986			H				H
Mennigmann and Lange, 1986	L	H	H			L	
Lapchine *et al.*, 1986, 1987, 1988; Moatti and Lapchine, 1986				L	L		
Manko *et al.*, 1987	L	L					H
Il'in, 1990				L			
Bouloc and D'Ari, 1991		—	—		—		
Pierson *et al.*, 1993			H				
Mennigmann and Heise, 1994	—	H			—	H	—
Gasset *et al.*, 1994	L	—					
Klaus, 1994; Klaus *et al.*, 1994, 1997	L	—	H	L			H
Tixador *et al.*, 1994	L	—	—	L	L		—
Horneck *et al.*, 1996					—		
Thévenet *et al.*, 1996	L						—
Kacena *et al.*, 1997; Kacena and Todd, 1997	L	—	—				
Lam *et al.*, 1999			L			H	

H - Parameter was higher on orbit; L - Parameter was lower on orbit; — - No change on orbit

2.4 BACTERIAL GROWTH

Bacterial growth is generally divided into three categories. Initially there is a lag phase when the bacteria do not grow. This is followed by an exponential growth

phase when the bacteria double every few hours or less. Eventually the bacteria stop growing, and the cell density remains relatively constant -- the stationary phase.

A number of studies have investigated the effects of acceleration on each of these phases. The results of experiments in space are briefly summarized in Table 2-1. Table 2-2 summarizes these experiments in more detail, and also includes the results from centrifuge and clinostat investigations of bacterial growth.

Although there are some conflicting reports, most studies have shown that modified inertial acceleration results in significant changes in the growth kinetics of bacteria. These reports have indicated that suspended bacterial cultures have a shorter lag phase and a higher final cell density on orbit when compared with ground controls. In a hypergravity environment, the lag phase has been found to be similar or slightly longer than controls, and the final cell density was equivalent or significantly lower than controls.

2.4.1 Length of Lag Phase

Many investigations of bacterial growth in space have indicated space flight results in a shorter lag phase for suspended bacterial cultures. Klaus *et al.* (1994, 1997) found the lag phase for suspended cultures of *E. coli* on orbit were 4 to 8 hours shorter than for cultures on the ground. Although it was not directly reported as such, growth curves for two other experiments with *E. coli* indicated the possibility of a shorter lag phase on orbit (Gasset *et al.*, 1994; Tixador *et al.*, 1994). A shorter lag phase for suspended cultures of *B. subtilis* on orbit was also reported by Mennigmann

Table 2-2 Effects of Inertial Acceleration on Bacterial Growth

Reference	Findings
Montgomery *et al.*, 1963	A 78% lower final cell density for *E. coli* at 1,000 *g* compared with controls. At 110,000 g, *E. coli* had a 98% lower final cell density, a 2 hour shorter lag phase, and a lower growth rate than controls.
Mattoni, 1968	Higher cell densities for *E. coli* and *Salmonella typhimurium* on orbit.
Kordium *et al.*, 1980	Higher cell density for *Proteus vulgaris* on orbit.
Ciferri *et al.*, 1986	Increased cell growth of *E. coli* on orbit.
Mennigmann and Lange, 1986	Reported a shorter lag phase, higher maximum growth rate, and a higher final cell concentration for *B. subtilis* on orbit.
Manko *et al.*, 1987	Reported a shorter lag phase for *Proteus vulgaris* on orbit when grown on agar compared with similar ground controls.
Lapchine *et al.*, 1990	No difference in the final cell density for *E. coli* at 1, 2, 5, or 10 *g*.
Bouloc and D'Ari, 1991	No difference in *E. coli's* growth yield or mean cell mass (from which they deduced the growth rate was unaltered) in space, 1 *g*, 3 *g*, or 5 *g*.
Pierson *et al.*, 1993	Total counts of clinically significant bacteria in urine samples from 144 astronauts were higher post-flight than preflight.
Mennigmann and Heise, 1994	No difference in the lag phase and approximately twice as high a maximum growth rate for *B. subtilis* in reduced gravity.
Gasset *et al.*, 1994	Possibility of a shorter lag phase for *E. coli* on orbit. Reported no change in the growth rate on orbit, yet also reported the doubling time was 22%* less in flight.
Klaus, 1994; Klaus *et al.*, 1994, 1997	The lag phase for *E. coli* was 4 - 8 hours shorter; the exponential phase was 2 - 5 hours longer; the growth rate was unchanged (or slightly lower), and the final cell density averaged 72%* higher on orbit.
Tixador *et al.*, 1994	The growth curves for *E. coli* in flight indicated a shorter lag phase.
Thévenet *et al.*, 1996	Found a shorter lag phase for a non-motile strain of *E. coli* on orbit. For a motile strain of *E. coli*, the lag phase was not affected in flight.
Kacena *et al.*, 1997; Kacena and Todd, 1997	Reported a shorter lag phase for *E. coli* and *B. subtilis* on an agar medium in space. *E. coli* also had a shorter lag phase during clinorotation on agar. No change in the final density was found.
Klaus *et al.*, 1998b	Clinorotation of *E. coli* resulted in a shorter lag phase and approximately twice as high of final cell density as static controls.
Lam *et al.*, 1999	Fewer viable cells of *Streptomyces plicatus* on orbit, yet they produced more of the antibiotic actinomycin D.

* Indicates statistically significant ($p < 0.05$).

and Lange (1986). In contrast to these results, however, a follow-on experiment did not confirm any change in the lag phase of *B. subtilis* in flight (Mennigmann and Heise, 1994).

Thévenet *et al.* (1994) also reported a shorter lag phase for suspended bacterial cultures on orbit. Similar to other investigations, their experiment used a non-motile strain of *E. coli*. However, they also found space flight did not affect the lag phase of a motile strain of *E. coli*. This was significant because previous experiments, using non-motile bacteria in liquid media, allowed the bacteria to sediment in the ground controls. Because motile bacteria are much less susceptible to the short-term effects of sedimentation, the experiment by Thévenet *et al.* indicated the absence of sedimentation on orbit could help explain the shorter lag phase for suspended bacterial cultures.

These results were somewhat contradicted by two experiments using semi-solid agar media. These investigations on agar, which also did not permit sedimentation, reported a shorter lag phase for *Proteus vulgaris* (Manko *et al.*, 1987), *E. coli,* and *B. subtilis* (Kacena and Todd, 1997) on orbit. However, due to a limited number of samples the *E. coli* and *B. subtilis* growth curves could have also been interpreted as having a slower growth rate on orbit, with no change in the lag phase.

In addition to these space experiments, ground experiments have also simulated the lack of sedimentation on orbit using a clinostat. In one experiment *E. coli* was cultured in a liquid medium (Klaus *et al.*, 1998b), and in the other *E. coli* and *B. subtilis* were grown on agar (Kacena and Todd, 1997). The experiments in a liquid

medium averaged approximately a 5 hour shorter lag phase than static controls. The experiments with *E. coli* on agar also reported a shorter lag phase during clinorotation. However, the clinostat experiments with *B. subtilis* on agar did not have a significant change in the lag phase during clinorotation.

In contrast to these experiments at hypogravity (real and simulated), which have generally reported a shorter lag phase, an experiment at 110,000 *g* found *E. coli* had longer lag phase relative to 1 *g* controls. At this level of acceleration *E. coli* had approximately a 2 hour longer lag phase than controls. However, when the acceleration was reduced to 1,000 g, the lag phase was not affected (Montgomery *et al.*, 1963).

2.4.2 Growth Rate

Studies of the growth rate during the exponential phase have yielded more controversial results. Five investigations reported little to no change in the growth rate on orbit; two others reported the growth rate to be higher in flight, and one experiment indicated space flight resulted in a lower growth rate. Experiments at hypergravity have reported either a lower growth rate or no significant change in the growth rate.

The first reported investigation of bacterial growth rate on orbit showed a higher maximum growth rate for *E. coli* compared with 1 *g* controls (Mennigmann and Lange, 1986). In a follow-on experiment, Mennigmann and Heise (1994) reported the growth rate for *B. subtilis* on orbit was initially similar to that of ground controls, but later increased to about twice the growth rate in the control samples.

Gasset *et al.* (1994) also found a higher growth rate (and lower doubling time) for *E. coli* on orbit. They reported a 22% lower doubling time on orbit, which was statistically significant. Surprisingly though, because the cells were not larger in size, as they expected for faster growing cells (Nanninga, 1985), they concluded that "microgravity has no effect on the growth rate" of *E. coli* (Gasset *et al.*, 1994).

There are two concerns with this conclusion. First, while Nanninga (1985) did show faster growing cells are larger, he pointed out that this relationship only held if the growth rate was greater than one doubling per hour. For growth rates less than this, Nanninga reported that the average cell size is constant. Because Gasset *et al.* (1994) had doubling times at or close to this threshold (1 doubling/hour for ground controls and 1.3 doublings/hour for flight) the relationship between cell size and growth rate may not have been valid. In addition, an experiment by Manko *et al.* (1987) indicated cell size was affected by mechanisms other than growth rate while in orbit (possibly reduced gravity). Their experiment using *P. vulgaris* on agar found a considerably lower growth rate on orbit. However, the cells were also larger when grown on orbit, with a slight decrease in their length-to-diameter ratio compared with ground controls. Considering these results, and the low growth rates reported by Gasset *et al.* (1994), their indirect method of measuring growth rate based upon cell size may not have been accurate. Their direct measurement, which indicated a significantly higher growth rate on orbit, was possibly a better indication of the actual growth rate.

In addition to these experiments that indicated growth rate was higher on orbit, a number of investigations have also found no change in the growth rate for bacteria in flight. Tixador *et al.* (1994) found a similar growth rate for *E. coli* on orbit. Klaus *et al.* (1997) also concluded "the rate of growth was unaffected (or slightly slower)" on orbit for *E. coli*. In addition, they reported that the duration of the exponential phase was two to five hours longer on orbit than it was for similar experiments on Earth.

Bouloc and D'Ari (1991) also reported no change in the growth rate for flight samples of *E. coli*. However, they did not measure growth rate directly. Similar to Gasset *et al.* (1994) they estimated growth rate by measuring the cell volume at a single point in time.

In addition to these experiments using liquid media, Kacena and Todd (1997) reported no difference in the growth rate on orbit for *E. coli* or *B. subtilis* when grown on an agar medium.

These inconsistent reports of the effect of microgravity on bacterial growth rate are most likely due to the constraints associated with space experiments. Size limitations often precluded the collection of multiple bacterial counts at different times during the relatively short exponential growth phase. This resulted in wide margins of error when evaluating the growth rate, μ.

Investigations of hypergravity's effects on bacterial growth have reported no change in the growth rate for 2, 5, or 10 *g* (Lapchine *et al.*, 1990) or for experiments at 1,000 *g* (Montgomery *et al.*, 1963). However, at accelerations of 110,000 g, the

growth rate was much less than controls, and the length of the exponential phase was longer than in experiments at 1 *g* (Montgomery *et al.*, 1963).

2.4.3 Final Cell Density

There have been a number of investigations of the effects of inertial acceleration on final cell density. Most of these experiments were conducted in space and reported a higher final density for suspended cultures (Ciferri *et al.*, 1986; Klaus *et al.*, 1997, 1994; Klaus, 1994; Kordium *et al.*, 1980; Mattoni, 1968; Mennigmann and Lange, 1986; Pierson *et al.*, 1993). One of the largest investigations involved 40 flight experiments with *E. coli* on 7 different shuttle missions (Klaus *et al.*, 1994, 1997). These experiments averaged a 72% increase in final cell density relative to ground controls ($p < 0.05$). As shown in Table 2-2, other experiments have also reported a higher final cell density on orbit for *E. coli* (Mattoni, 1968), *B. subtilis* (Mennigmann and Lange, 1986), *P. vulgaris* (Kordium *et al.*, 1980), and *S. typhimurium* (Mattoni, 1968). In addition to these *in vitro* studies, *in vivo* experiments have found similar results. Pierson *et al.* (1993) reported higher average bacterial levels in post-flight analysis of urine samples from 144 astronauts compared with their preflight samples.

In contrast to these experiments reporting a higher final cell density in flight, one experiment with *Streptomyces plicatus* found fewer viable cells on orbit. However, the space samples produced higher levels of antibiotics on orbit (Lam *et al.*, 1999). A few other investigations reported that final cell density was not affected on orbit (Bouloc and D'Ari, 1991; Kacena *et al.*, 1997; Tixador *et al.*, 1994). Of particular interest, Kacena *et al.* (1997) conducted the only investigation of final cell

density using a semi-solid agar medium. Their experiments with *E. coli* and *B. subtilis* on four shuttle flights found no difference in the final cell population on orbit when compared with ground samples.

There have also been a few ground studies using a clinostat to simulate the lack of sedimentation and convection on orbit. These experiments found results similar to those from flight (Mattoni, 1968; Mennigmann, 1994, Klaus *et al.*, 1998b, Kacena and Todd, 1997). Klaus *et. al.* (1998b) reported that clinorotation of *E. coli* resulted in approximately twice as high a final cell density relative to static controls. A separate experiment on agar found the final cell densities for *E. coli* and *B. subtilis* were not affected by clinorotation (Kacena and Todd, 1997).

Experiments at accelerations greater than 1 *g* have resulted in either no change in final cell density or drastically lower final cell concentrations, depending upon the level of acceleration. Lapchine *et al.* (1990) cultivated suspended cultures of *E. coli* over a 9 hour period at 1, 2, 5, and 10 *g*. They found no difference in the final cell densities at these accelerations. This was supported by a second investigation that concluded accelerations of 3 *g* or 5 *g* did not affect *E. coli's* final cell density (Bouloc and D'Ari, 1991). Montgomery *et al.* (1963) found significantly different results for *E. coli* when grown at high accelerations. When centrifuged at 1,000 *g*, the final cell densities averaged 78% lower than static controls. When the acceleration was increased to 110,000 *g*, final cell densities were 98% lower on average than in control samples.

2.4.4 Summary of Bacterial Growth

In summary, there have been a number of growth experiments in space and a few investigations of bacterial growth using a clinostat. There have also been three reports of bacterial growth using a centrifuge. The most consistent results have indicated that acceleration affects the lag phase and final cell density of suspended bacterial cultures. Table 2-3 summarizes these results.

Table 2-3 Summary of Altered Growth Due to Inertial Acceleration. The numbers shown are relative to static controls and were taken from the largest reports using suspended bacterial cultures.

Acceleration	Lag Phase	Final Cell Density
Space Flight (Klaus *et al.*, 1997)	4-8 hours shorter	72% higher
Clinostat (Klaus *et al.*, 1998b)	5 hours shorter	~100% higher
2, 5, and 10 *g* (Lapchine *et al.*, 1990)	not measured	no change
1,000 *g* (Montgomery *et al.*, 1963)	no change	78% lower
110,000 *g* (Montgomery *et al.*, 1963)	2 hours longer	98% lower

A majority of investigations have reported that hypogravity (real and simulated) reduced the lag phase for suspended cultures (Klaus *et al.*, 1997; 1998b). There have also been two reports that space flight resulted in a shorter lag phase for bacteria cultivated on agar (Manko *et al.*, 1987; Kacena and Todd, 1997). One noteworthy exception indicated the lag phase of motile bacteria was unaffected by microgravity conditions (Thévenet *et al.*, 1996). Experiments at hypergravity have

reported the lag phase for *E. coli* was unaffected at 1,000 *g*, and averaged 2 hours longer than controls when centrifuged at 110,000 *g* (Montgomery *et al.*, 1963).

It has also been consistently reported that hypogravity (real and simulated) results in almost twice as high a final cell density for suspended bacterial cultures (Klaus *et al.*, 1997; 1998b). For cultures grown on agar, though, the final density was not affected by space flight (Kacena *et al.*, 1997; Kacena and Todd, 1997). Hypergravity experiments also resulted in no change in the final cell density for *E. coli* at accelerations up to 10 *g* (Lapchine *et al.*, 1990). However, experiments at 1,000 g, averaged a 78% lower final cell density than controls, and experiments at 110,000 *g* averaged a 98% lower final density (Montgomery *et al.*, 1963). Most of these observations have been confirmed at growth temperatures of approximately 37°C and 23°C.

2.4.5 Theories of Bacterial Growth

Almost all of the explanations for altered bacterial growth have focused on the experimental results from space. The hypotheses for these observed changes can be broken into two groups. A few theories postulate that gravity affects bacteria "directly" (Bouloc and D'Ari, 1991; Mesland, 1987; Schatz *et al.*, 1994). Other hypotheses argue that that gravity "indirectly" affects suspended bacterial cultures through changes in their surrounding fluid environment (Bjorkman, 1988; Klaus *et al.*, 1997; Mattoni, 1968; Thévenet *et al.*, 1996).

One theory, suggesting gravity directly affects bacterial cells, proposes "the faster proliferation in space is due to an economy of cell energy normally expended on

'uphill' mobility" (Bouloc and D'Ari, 1991). Similar theories argue that reduced gravity causes small changes in the "cellular machinery" (Mesland, 1987) or the cell membrane (Schatz *et al.*, 1994), which alters the cell's energy requirements and stimulates growth on orbit.

In contrast to these theories, the hypothesis that gravity "indirectly" affects bacterial cells argues that in a 1 *g* environment cells slowly sediment, eventually resulting in a high concentration of cells and waste at the bottom of a culture tube. This "less than ideal" growth environment does not occur in flight, where cells remain evenly distributed in the liquid medium. This allows more efficient nutrient transfer, and lower levels of waste around each cell, resulting in a higher final cell concentration on orbit (Klaus *et al.*, 1997; Mattoni, 1968; Thévenet *et al.*, 1996).

This model of "indirect" effects of gravity is also used to explain the shorter lag phase observed with suspended cultures in space. It is postulated that the lack of sedimentation on orbit allows bacteria to remain near their by-products in flight. Some of these by-products are believed to be beneficial to growth (possibly cofactors or enzymes). Because bacteria on orbit remain surrounded by these by-products, they come out of lag phase sooner than ground controls. In contrast, it is postulated that cells on Earth sediment away from these beneficial by-products, and as a result require more time to come out of lag phase (Klaus *et al.*, 1997).

Most experiments support the theory that gravity indirectly affects the growth of suspended bacterial cultures. For example, Kacena *et al.* (1997) found no difference in the final cell densities for *E. coli* or *B. subtilis* on orbit when they were

grown on a semi-solid agar medium. Another experiment found no difference in the lag phase for a motile strain of *E. coli* on orbit (Thévenet *et al.*, 1996). Because neither of these experiments were affected by sedimentation, they both indicated reduced gravity only "indirectly" affected bacterial growth, through sedimentation and fluid changes surrounding the cells. Thévenet *et al.* (1996) concluded that it is possible that the absence of gravity allows the accumulation of a small "cloud" of dissolved carbon dioxide around the bacterial cells, which allows them to come out of lag phase sooner than 1 *g* controls. This is consistent with laboratory studies that have shown a lack of dissolved carbon dioxide in the medium can increase the lag phase (Barford *et al.*, 1982).

Clinostat experiments with suspended cultures have also supported this theory. These experiments by different research groups had similar results to space flight -- a shorter lag phase and a higher final cell density than controls (Mattoni, 1968; Mennigmann, 1994, Klaus *et al.*, 1998b). Because a clinostat only simulates the "indirect" effects of reduced gravity, not the "direct" effects, these findings support the theory that gravity affects suspended bacterial cultures indirectly through changes in their surrounding fluid environment.

The idea that gravity indirectly affects bacteria was originally proposed almost 30 years ago (Mattoni, 1968). However, not until 1997, was a physical mechanism identified that could explain how this was achieved (Klaus *et al.*, 1997). This idea of bacteria sedimenting toward the bottom of a test tube could explain the shorter lag phase and higher final cell density observed in hypogravity. However, it can not

directly explain the results at hypergravity. At high accelerations, bacteria quickly become concentrated at the bottom of the test tube. According to the proposed theory, this concentration of cells should initially be beneficial to growth. Because the bacteria are surrounded by excreted by-products from many cells, they should come out of lag phase much sooner than controls. However, this is not what Montgomery *et al.* (1963) found with their two hypergravity experiments.

Although it has been 35 years since the experiments by Montgomery *et al.* (1963), no one has yet proposed a physical mechanism that can explain their results (Moore and Cogoli, 1996). It has been postulated that there could have been a direct effect on the cells by the high acceleration forces. However, other research showed that high accelerations did not disrupt the cellular structure of similar cells (Montgomery *et al.*, 1963) or change the DNA, RNA, or protein synthesis of *E. coli* (Pollard, 1971).

In summary, the majority of evidence indicates that acceleration affects bacterial growth through changes in the fluid environment surrounding each bacterium. It has been proposed that sedimentation of the bacterial cells causes these fluid changes. While this could possibly explain the observed shorter lag phase and higher final cell density observed in space, this theory can not directly explain the results of bacterial growth at hypergravity.

Investigations of crystal growth on orbit have identified another gravity-dependent force that affects the fluid environment surrounding crystals (Brailovskaya *et al.*, 1994; Fehribach and Rosenberger, 1989; McCay and McCay, 1994; Pusey and

Naumann, 1986; Pusey *et al.*, 1988, 1986). As a crystal grows it depletes solute from the medium immediately surrounding the crystal. This creates density differences, which in a 1 *g* environment cause small convection currents around the crystal. The research in this dissertation proposes that this same physical mechanism could affect bacterial growth as the cells consume glucose from their surrounding medium. This model is explained and investigated in detail in Chapters 4 through 7.

2.5 ANTIBIOTIC EFFECTIVENESS

In addition to higher cell densities on orbit, investigations have also clearly shown that antibiotics are less effective in space (see Table 2-4). This is particularly troubling for manned space missions, considering the number of investigations that also show the human immune system is depressed in space (Cogoli *et al.*, 1984; Mesland, 1987; Konstantinova, 1991). The combination of higher bacterial growth, repressed immune systems, and less effective antibiotics poses a serious problem for long duration manned space missions.

These effects of space flight could explain reports of diarrheal illness on the space shuttle and at least two life-threatening infections during space missions, one Soviet and one U.S. (Meehan *et al.*, 1993). In one case, the long duration Soviet mission on Salyut-7 had to be terminated early because of "acute inflammatory disease" (Mennigmann, 1994).

Table 2-4 Effects of Inertial Acceleration on Antibiotic Effectiveness

Reference	Findings
Taylor and Zaloguev, 1978	Antibiotics were less effective on bacteria collected in flight from the bodies of Apollo-Soyuz astronauts and cosmonauts compared with similar samples collected preflight and post-flight.
Tixador *et al.*, 1981, 1985; Lapchine *et al.*, 1987	The MICs (Minimal Inhibitory Concentrations) for colistin and kanamycin were over 4 times* higher on orbit for *E. coli*. Chloramphenicol and erythromycin had higher MICs (0-100%) for *Staphylococcus aureus*. However, in every case the decreased antibiotic effectiveness was temporary and disappeared in post-flight cultures.
Lapchine *et al.*, 1986, 1987, 1988; Moatti and Lapchine, 1986	The MIC for colistin was almost double for flight cultures of *E. coli*. The flight samples had over 100 times* more living cells than controls with the same level of antibiotics. No differences in antibiotic effectiveness were found between the flight samples in reduced gravity and flight samples on a 1 *g* centrifuge.
Il'in, 1990	More bacterial resistance to tetracycline was found in post-flight samples from the Salyut-7 crew compared with preflight samples.
Lapchine *et al.*, 1990	No difference in the effectiveness of dihydrostreptomycin at subinhibitory levels on *E. coli* in 1, 2, 5, or 10 *g* ground studies.
Ilyin, 1992	Studies of antibiotic sensitivity with cosmonauts on 5 space flights had similar results to confined ground simulations.
Tixador *et al.*, 1994	*E. coli* cultures with the antibiotic dihydrostreptomycin had a higher growth rate on orbit. There was also a higher MIC for in-flight cultures. Experiments on orbit in a 1 *g* centrifuge did not show any differences from those in microgravity.
Klaus, 1994	The effectiveness of three antibiotics against which *E. coli* can develop specific resistance mechanisms (nalidixic acid, gentamicin, and erythromycin) was significantly reduced on orbit.* Other antibiotics, against which *E. coli* uses non-specific resistance mechanisms (colistin and actinomycin D), did not have a lower effectiveness in flight.

* Indicates statistically significant ($p < 0.05$).

2.5.1 *In vivo* Studies of Antibiotic Effectiveness

Two *in vivo* studies have highlighted this dilemma. During the Apollo-Soyuz Test Project, Zaloguev recovered samples of microflora on various parts of crewmembers' bodies. Bacterial samples collected from astronauts and cosmonauts preflight, in flight, and post-flight were tested with several antibiotics after the mission. They found antibiotics were less effective against bacteria collected during flight than they were against samples collected preflight or post-flight (Taylor and Zaloguev, 1978). In a similar study, Il'in (1990) reported that the majority of coliform bacterial cultures sampled post-flight from the Salyut-7 crew were resistant to tetracycline, yet there had been no resistant strains in the preflight samples.

Typical of *in vivo* studies, these results could have been caused by a number of factors. They may not have been due solely to less effective antibiotics in space. The results might have also been caused by more bacterial growth on orbit, a repressed human immune system, or the confined environment associated with manned space missions.

A ground study by Polikarpov and Bragina (1989) illustrated this last possibility. In their study, 14 individuals were confined in an airtight container for 6 to 175 days. Following their confinement, 11 subjects had an increase in antibiotic resistant microorganisms compared with their pre-confinement levels. Another study by Ilyin (1992), showed similar trends in the levels of antibiotic sensitivity for cosmonauts in space and in confined ground simulations. Both of these findings indicated that bacteria are more likely to become resistant to antibiotics simply because

space crews are in confined environments with other individuals. This illustrates the difficulty with *in vivo* studies on orbit. It is extremely difficult to determine if observed changes are due to reduced gravity or if they are caused by other variables associated with space flight.

2.5.2 *In vitro* Studies of Antibiotic Effectiveness

In vitro studies involve fewer variables and are more suited to quantitatively analyzing the effectiveness of antibiotics in space. These experiments have also indicated antibiotics are less effective on orbit.

In 1982, the Cytos 2 experiment on Salyut-7 found the Minimal Inhibitory Concentrations (MIC) for colistin and kanamycin were over four times higher for in-flight cultures of *E. coli* when compared with ground samples ($p < 0.05$). The MICs were also higher for chloramphenicol and erythromycin in cultures of *Staphylococcus aureus* on orbit. In both of these cases, the decreased antibiotic effectiveness was not permanent. Cultures grown post-flight from the flight samples did not show any increased resistance to antibiotics (Lapchine *et al.*, 1987; Tixador *et al.*, 1985, 1981).

In a follow-up experiment on STS 61-A, the MIC for colistin was twice as high for cultures of *E. coli*. There were also over 100 times more living cells in the flight samples given sub-inhibitory levels of antibiotics, compared with similar ground controls ($p < 0.05$) (Lapchine *et al.*, 1987, 1988; Moatti and Lapchine, 1986).

A third experiment by the same research group was conducted in 1992 on the IML-1 mission. On this flight, they found a higher MIC for dihydrostreptomycin in in-flight cultures of *E. coli*, and a higher growth rate in cultures using a sub-inhibitory

level of antibiotics (Tixador *et al.*, 1994). Using this same antibiotic with cultures of *E. coli*, Lapchine *et al.* (1990) found no difference in the MIC for ground experiments at 2, 5, or 10 *g* compared with 1 *g* controls.

Experiments by Klaus (1994) with *E. coli* also showed a reduced effectiveness for some antibiotics on orbit. His experiment divided antibiotics into two categories -- those antibiotics against which *E. coli* can develop specific resistance mechanisms (nalidixic acid, gentamicin, and erythromycin) and those against which *E. coli* uses non-specific resistance mechanisms (actinomycin D and colistin). The three antibiotics in the first group were all significantly less effective on orbit, while the two antibiotics that can only be resisted by non-specific mechanisms were not affected on orbit.

2.5.3 Theories of Antibiotic Effectiveness

Different theories have been proposed to explain the reduction of antibiotic effectiveness on orbit. While each hypothesis explains some findings, most theories can not explain the results of all experiments.

The simplest explanation is that the lower antibiotic effectiveness on orbit is not as much a result of less effective antibiotics, but primarily a consequence of the higher cell growth on orbit (Tixador *et al.*, 1992, Lapchine *et al.*, 1988). Because suspended bacterial cultures start growing sooner and grow to a higher level on orbit, there are simply more bacteria to kill in the flight samples. This explanation, however, does not explain why Klaus (1994) found the effectiveness of two antibiotics, which can only be resisted using only non-specific mechanisms, were not affected on orbit.

Another explanation for the reduced effectiveness of antibiotics in space was identified by Ciferri *et al.* (1986) in an investigation of the three basic methods of exchanging genetic information between *E. coli* on orbit. These mechanisms are: conjugation (cell-to-cell interaction), transduction (bacteriophage-to-cell interaction), and transformation (free DNA fragments-to-cell interaction). For conjugation, they found no difference in the transmission rate for the first marker. However, later markers appeared to be transmitted more efficiently in reduced gravity, with three to four times as many recombinants compared with ground controls and flight controls in a 1 *g* centrifuge. Even taking into account the higher cell concentration, the difference in the transmission of the later characters was still evident. Ciferri *et al.* (1986) concluded that cell-to-cell interactions were less likely to be disrupted in microgravity. This improved conjugation could help explain the increased drug resistance observed in space. Mutation is generally the cause for acquiring resistance to antibiotics, but conjugation is also recognized as extremely important for spreading DNA sequences that can confer resistance to antibiotics (Gilman *et al.*, 1985). Through enhanced conjugation, bacteria could more efficiently pass information for resisting antibiotics in flight. This explanation does not, however, explain why antibiotic effectiveness returns to preflight levels after returning from space. Additionally, the growth kinetics of cells was not consistent with the emergence of genetically resistant clones.

Another explanation for the reduced antibiotic effectiveness on orbit was proposed by Lapchine *et al.* (1988). They postulated that some antibiotics may be less effective in space due to modifications in the cellular envelope (thickness or

permeability). Their finding that in-flight cultures of *S. aureus* had "a very marked increase of the thickness of the cell wall" supported this theory (Lapchine *et al.*, 1986, 1987; Tixador *et al.*, 1981, 1985). However, on the same flight, they also found no change in the cell wall thickness of *E. coli* cells. In addition, more recent experiments reported no changes in the cell wall thickness of *E. coli* (Tixador *et al.*, 1994) or *B. subtilis* (Mennigmann and Heise, 1994) on orbit.

It has also been proposed that space radiation causes the reduced antibiotic effectiveness on orbit. This theory was supported by experiments on Spacelab D-1 (Lapchine *et al.*, 1987, 1988; Moatti and Lapchine, 1986) and the IML-1 mission (Tixador *et al.*, 1994). Both experiments reported a similar reduction in antibiotic effectiveness for cultures in microgravity and in a 1 *g* centrifuge on orbit. These findings, combined with the hypergravity experiments by Lapchine *et al.* (1990), led them to conclude that space flight factors other than inertial acceleration caused the reduced antibiotic effectiveness. They, therefore, proposed that "an effect of cosmic radiation" was the best explanation for their observed results (Tixador *et al.*, 1994).

It is important to point out, however, that the 1 *g* in-flight controls for these two experiments were not placed on the centrifuge until five days (Lapchine *et al.*, 1988) or eight days (Tixador *et al.*, 1994) after launch. As another researcher using the same equipment noted, because the "controls were not placed on the centrifuge during the long storage period" they could have "been influenced by possible microgravity effects" (Gasset *et al.*, 1994). In addition to these concerns, the theory that radiation causes antibiotics to be less effective on orbit can not explain why two

antibiotics tested by Klaus (1994) were not affected in space. Furthermore, there is no known evidence from Earth-based research that a constant ionizing radiation dose below 10^{-2} Gy, which is typical of space radiation (Badhwar *et al.*, 1992), can induce mutation rates toward antibiotic effectiveness.

A final explanation for the reduced effectiveness of antibiotics in space relates to the colloidal state of bacterial cells on orbit (Klaus *et al.*, 1997). As bacteria sediment through a culture on Earth, they encounter antibiotics. On orbit, however, because they do not sediment they encounter antibiotics at a slower rate. It has been postulated that this could allow bacteria to increase their antibiotic resistance in flight. This explanation can explain the unique results of Klaus (1994), who tested a variety of antibiotics. For three antibiotics, against which *E. coli* develops specific resistance mechanisms, the slower rate of antibiotic contact could allow bacteria more time to develop resistance mechanisms. It is expected that these antibiotics would therefore be less effective on orbit. However, for the two antibiotics against which *E. coli* uses non-specific resistance mechanisms, the slower antibiotic contact would not be beneficial, and antibiotics would be just as effective on orbit. This was exactly what Klaus (1994) observed. This explanation is also consistent with the reports that antibiotic effectiveness returns to preflight levels after returning from space.

2.6 EFFECTS OF RADIATION

Many experiments have been performed to study the effects of radiation on microorganisms in space. Almost all of these investigations have found radiation had

almost no effect on microorganisms inside a space vehicle. Before the dawn of the space age, over 32 high altitude balloon experiments and a few rockets were used to study space radiation (Beischer and Fregly, 1962; Kern and Hock, 1993). They found that direct exposure of microorganisms to space radiation caused severe damage or death. However, with aluminum shielding no unusual effects were noted in a 1 *g* space environment compared with ground controls.

Experiments at the beginning of the space age had similar results. Yuri Gagarin's first manned space mission in 1961, a Mercury mission, and three Gemini missions all concluded there were no effects due to radiation provided the samples were inside a spaceship (Kern and Hock, 1993; Zhukov-Verezhnikov, 1962).

More recent experiments have gone beyond the question of whether bacteria can survive in space (see Table 2-5). They have addressed the possibility that space radiation might affect specific characteristics of bacteria on orbit. For example, when Mennigmann and Heise (1994) reported higher bacterial growth rates on the IML-1 mission, their controls were placed in an on-board centrifuge. This allowed them to rule out the effects of radiation as one of the non-inertial variables associated with space flight. They concluded the altered growth kinetics on orbit were due to the reduced gravity environment of space, not to other factors such as space radiation.

Table 2-5 Recent Radiation Experiments with Bacteria in Space

Reference	Findings
Lapchine *et al.*, 1986, 1987, 1988; Moatti and Lapchine, 1986	Flight samples in reduced gravity and on a 1 *g* centrifuge both had reduced antibiotic effectiveness relative to ground controls. This indicated some non-inertial effect of space, such as radiation, could have caused the decrease in antibiotic effectiveness.
Bouloc and D'Ari, 1991	No significant DNA damage to *E. coli* from radiation.
Mennigmann and Heise, 1994	The growth rate of *B. subtilis* on orbit was higher than 1 *g* controls on an in-flight centrifuge or similar ground controls. Samples on the in-flight centrifuge had similar growth rates as the ground controls. They, therefore, concluded that the altered growth rate on orbit was not caused by non-inertial factors of space flight, such as radiation.
Tixador *et al.*, 1994	Found a similar reduction in antibiotic effectiveness for flight samples in reduced gravity and on a 1 *g* centrifuge when compared with ground controls. This lead them to speculate that a non-inertial factor of space flight, such as radiation, might have affected the samples on orbit.
Horneck *et al.*, 1996	No differences noted in flight and ground samples of *E. coli* and *B. subtilis* to repair radiation-induced DNA damage.

Higher growth rates and increased cell volume of *Paramecium tetraurelia* have also been reported on orbit, yet were determined to not be a result of launch vibrations or cosmic radiation (Planel *et al.*, 1982). Other experiments have found radiation did not cause significant DNA damage to *E. coli* in space (Bouloc and D'Ari, 1991), and cells could repair radiation-induced DNA damage as well on orbit as they can on Earth (Horneck *et al.*, 1996).

The only two recent studies that claimed radiation might have affected bacterial samples were on Spacelab D-1 (Lapchine *et al.*, 1987, 1988; Moatti and Lapchine, 1986) and the IML-1 mission (Tixador *et al.*, 1994). These experiments, which were discussed in the previous section, reported a similar reduction in antibiotic effectiveness for cultures in microgravity and in a 1 *g* centrifuge on orbit. Although the authors concluded that radiation might have caused the lower antibiotic effectiveness in space, as previously mentioned, their results could have been due to a long storage period in microgravity (5 to 8 days) before the samples were placed in the centrifuge.

In addition, measurements of the ionizing radiation doses encountered in space are not expected to affect bacteria. For example, the total dose measured over a 4-day period on shuttle flight STS-31 (launch of the Hubble Space Telescope), was between 6.2 and 11.6 mGy (Badhwar *et al.*, 1992). This mission included 25 passes through the South Atlantic Anomaly at high (620 km) altitudes, where relatively high radiation doses are present. However, it was still much less than required to affect bacteria. In comparison, the most radiation sensitive bacteria are responsive (reproductive death) to minimal doses around 1.0 Gy, about 100 times a typical mission dose (Kaplan and Moses, 1964; Sparrow *et al.*, 1967; Todd, 1994). Gene-specific mutations require yet higher doses. It is, therefore, very doubtful that radiation affects bacterial experiments in space.

2.7 PRODUCTION OF SPORES AND SECONDARY METABOLITES

It addition to the observed changes in growth kinetics and antibiotic effectiveness, space flight has also been reported to enhance the production of secondary metabolites. This is particularly interesting because of its potential economic impact. A better understanding of the mechanisms affecting metabolite production could lead to economically viable bioprocessing on orbit or allow more efficient terrestrial bioprocessing.

This section summarizes the few experiments in which secondary metabolite production in various inertial environments has been investigated. An extensive literature review revealed only one investigation of bacterial metabolism has been accomplished in space. There have also been three ground experiments using bacteria in a rotating bioreactor that reduces shear forces in an attempt to simulate some aspects of space flight. However, an extensive literature review found no metabolism experiments at acceleration levels greater than 1 *g*.

This section also reviews two experiments that have studied the production of spores on orbit. These experiments are included because it is believed that sporulation and secondary metabolite formation compete for common nutrients (Johns, 1992). The results from all of these experiments are briefly summarized in Table 2-6.

Table 2-6 Production of Spores and Metabolites by Bacteria in Different Inertial Environments

Reference	Findings
Experiments on Orbit:	
Mennigmann and Lange, 1986	Fewer cells of *B. subtilis* produced spores in flight.
Mennigmann and Heise, 1994	Four times higher proportion of spores for *B. subtilis* on orbit compared with ground and flight controls in a 1 *g* centrifuge. Spore survival rate was not affected on orbit.
Lam *et al.*, 1999	Fewer viable cells of *Streptomyces plicatus* were cultivated on orbit. However, when grown in a complex liquid medium the space samples averaged 115% higher maximum specific productivity of antibiotics.
Ground Experiments in NASA's "High-Aspect" Rotating Vessel (HARV):	
Fang *et al.*, 1997a	Growth of *Streptomyces clavuligerus* was 40% lower on NASA's HARV compared with controls. Also, the specific productivity of β-lactam was 59% lower.
Fang *et al.*, 1997b	Found no difference in the production of gramicidin S by *Bacillus brevis* or in the repressive effect of glycerol on NASA's HARV compared with 1 *g* controls.
Fang *et al.*, 1997c	Production of the antibacterial polypeptide microcin B17 by *E. coli* was inhibited. When grown in flasks, the microcin was cellular, but when grown in the HARVs almost all of the microcin was found in the medium.

2.7.1 Experiments on Orbit

The first experiment involving sporulation on orbit was conducted by Mennigmann and Lange in 1986. They reported substantially reduced spore formation on orbit by *B. subtilis* when compared with ground controls. The flight samples

produced only 5×10^4 spores/ml compared with 8×10^5 spores/ml for the ground samples. The number of cells that produced spores was also much lower on orbit. Only 1×10^5 cells/ml produced spores in flight, while 3×10^7 cells/ml produced spores on the ground.

A follow-on experiment by Mennigmann and Heise (1994) had drastically different results with *B. subtilis*. They reported the proportion of spores in flight was 60%, while ground controls and on-board 1 *g* controls had only a 15% proportion of spores. This second experiment also found the spores survived just as well on orbit as they did on the ground.

A much more recent experiment, in November 1996, examined the production of secondary metabolites by *Streptomyces plicatus* (Lam *et al.*, 1999). This experiment is believed to be the first, and only, test of secondary metabolite production by bacteria on orbit. Using a defined liquid medium and a complex liquid medium, actinomycin D was produced in flight and on the ground during the Space Shuttle mission STS-80. The bacteria grew for 7, 12, and 16 days in a dark, 22°C environment. Post-flight analysis revealed similar amounts of bacteria in the flight and ground samples. However, the same amount of bacteria produced different levels of antibiotics on orbit. HPLC analysis showed the specific productivity of actinomycin D (measured in μg/mg of extract) reached a maximum in the ground samples at day 7 in both the defined and complex media (reference Figure 2-1). The space samples, however, reached a maximum at day 12 in the complex media, and continued to rise in the defined medium. There was no difference in the maximum specific productivity

(ActD/weight) in space and on the ground using the defined medium. However, the complex medium had a 115% higher specific productivity on orbit.

Both media also had fewer viable cells in space after 16 days (measured in CFU/ml). However, samples in both media on orbit produced more antibiotics per CFU. After 16 days the defined medium had a 300% higher productivity per CFU on average, and the complex medium had 600% higher productivity per CFU than ground controls.

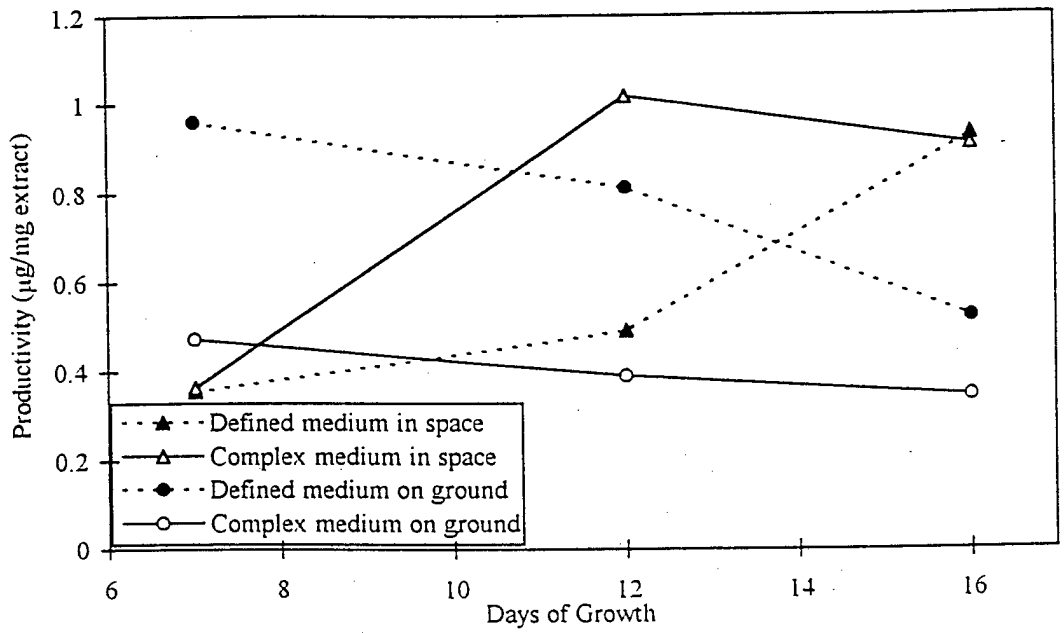

Figure 2-1 Specific Productivity of Actinomycin D on Orbit. Space samples had a higher specific productivity than ground controls after 16 days when using a complex or defined media. The maximum specific productivity was 115% higher in space for samples cultivated in the complex medium (Lam *et al.*, 1999).

A similar experiment was conducted by Lam *et al.* (1998) on STS-77, in May 1996. This earlier experiment also examined the production of secondary metabolites

on orbit. However, it differed in two ways from their experiment with *S. plicatus*. For this earlier experiment the producing microorganism was a fungus (*Humicola fuscoatra*), not a bacterium. It was also cultivated on agar, not in a suspended culture. Comparing the ground and flight samples from this experiment, both had similar levels of fungal growth. However, on two different semi-solid agar media, the flight samples produced more of the antibiotic monorden. When the fungus was grown on T8 agar, the average flight sample produced 30% more monorden than the ground controls. When grown on a PG agar medium, the flight samples produced an average of 190% more antibiotics than the ground samples ($p < 0.05$). Further studies are necessary to confirm these results and to identify mechanisms responsible for these changes.

2.7.2 Ground Experiments

In contrast to these experiments in space, Fang *et al.* conducted ground experiments using a "high-aspect" rotating vessel (HARV) bioreactor. This special bioreactor attempts to simulate the microgravity environment by reducing shear forces to less than one-tenth the force present in typical bioreactors. In three experiments they found that less antibiotics were produced by *Streptomyces clavuligerus* (Fang *et al.*, 1997a), a similar amount of antibiotics were produced by *Bacillus brevis* (Fang *et al.*, 1997b), and the production of the antibacterial polypeptide microcin B17 was inhibited by *E. coli* (Fang *et al.*, 1997c).

These results suggest the HARV does affect metabolite production, but it does not produce consistent responses among microbial species. The HARV experiment with *Streptomyces* also did not produce results consistent with a similar experiment on

orbit (Lam *et. al.*, 1999). These differences could have been due to the shear forces created by the HARV. The HARV's results might have also been affected by the high rotational rate of 60 to 120 RPM, which created an estimated maximum centrifugal force of 0.2 to 0.6 *g*.

2.8 OTHER FINDINGS

To conclude this comprehensive review of bacterial studies in various inertial environments, a brief summary of miscellaneous experiments is provided in Table 2-7. Some of these experiments were discussed previously as they related to various theories involving bacterial growth and antibiotic resistance.

Table 2-7 Miscellaneous Bacterial Experiments at Different Levels of Acceleration

Reference	Findings
Mattoni, 1968	Lower yield of bacterial viruses on orbit.
Pollard, 1971	Found no difference in the ability of *E. coli* to synthesize DNA, RNA, or protein at 50,000 *g*.
Tixador *et al.*, 1981 1985; Lapchine *et al.*, 1987	Significantly thicker cell wall for *S. aureus* on orbit, but not for *E. coli*.
Ciferri *et al.*, 1986	Three to four fold increase in *E. coli* conjugation (cell-to-cell interaction) for late markers. No difference was found in the conjugation of early markers or in transduction (cell-to-bacteriophage interaction).
Manko *et al.*, 1987	An increase in swarming for *Proteus vulgaris* on orbit. The flight cells were also larger with a slight decrease in the length-to-diameter ratio.
Mennigmann and Heise, 1994	No changes in cell wall thickness, re-adaptation to exponential growth, genetic stability, or repair capacity of *B. subtilis* on orbit.
Klaus *et al.*, 1994	Found a difference in proteins expressed in the region of 20-30 kD for *E. coli*, and improved nutrient adaptation compared with ground controls.
Tixador *et al.*, 1994	Reported no difference in *E. coli's* cell envelope thickness on orbit compared with ground controls.
Thévenet *et al.*, 1996	The system of signal transduction across the *E. coli* envelope that involves osmoregulation was functional in microgravity.

2.9 CONCLUSIONS

There have been many contradictory findings of the effects of varying inertial acceleration on bacteria. In some specific areas, however, there have been consistent results, upon which general conclusions can be based. For example, most data indicate bacteria in suspended cultures have a shorter lag phase and higher final cell density on orbit. There is also evidence that very high accelerations can increase the length of the lag phase and also dramatically reduce the final cell density. The only proposed mechanism for any of these results argues that the lack of sedimentation of cells in space causes the observed shorter lag phase and higher final cell density. Unfortunately this mechanism can not directly explain the longer lag phase observed in hypergravity.

There is also consistent evidence that antibiotics are less effective on orbit. While there is still no explanation for these results, the most likely explanation is an indirect effect of reduced gravity.

In other areas, particularly the production of secondary metabolites, there is still insufficient data to draw broad conclusions. Only one experiment involving bacterial metabolism has been conducted in space, and no metabolism experiments have been accomplished at accelerations greater than 1 *g*. Due to the potential scientific discoveries and economic benefits, this area warrants further investigation in an attempt to identify an underlying physical mechanism.

CHAPTER 3

GENERAL MATERIALS AND METHODS

This chapter describes general materials and methods used in this dissertation, including a description of the cells and media, general hardware, and analysis methods. A few experiments have also used specific materials or methods, which are not covered in this chapter, but are discussed later in a more appropriate chapter. For example, a description of the unique hardware used for the flight experiment is covered in Chapter 7.

3.1 CELLS AND MEDIA

Escherichia coli strain ATCC 4157 (low-motility original Escherich strain) was used for all experiments. The bacteria were grown in fluid-filled culture tubes at an ambient temperature of $21 \pm 1\,^{\circ}C$. There was initially some dissolved oxygen in the medium, which was depleted by the beginning of the exponential phase of growth (E. Smith, University of Colorado, Boulder, CO, USA, 1998). Bacteria were cultivated in Vogel-Bonner "Medium E" minimal growth medium (Vogel and Bonner, 1956) containing approximately 5.9 ± 0.25 g/l of glucose. The inoculum of 10^6 cells/ml was taken from a saturated culture, grown in Medium E without glucose. This inoculation

procedure allowed a longer lag phase to be observed when the bacteria were transferred to a medium containing glucose.

One liter of 50x concentrated Medium E contained the constituents listed below.

Distilled Water	670 ml
$MgSO_4 \cdot 7H_2O$	10.0 g
citric acid$\cdot H_2O$	100.0 g
K_2HPO_4·anhydrous	500.0 g
$NaNH_4HPO_4 \cdot 4H_2O$	175.0 g

After this was prepared it was autoclaved. The medium was later diluted 49:1 in sterilized, distilled water with no glucose for the inoculum medium, or with 5.9 g/l of glucose for the growth medium.

3.2 HARDWARE

3.2.1 Culture containers

Two different culture containers were used -- a standard 9 ml screw-cap tube and a glass barrel used for flight experiments, called an FPA (Fluids Processing Apparatus). FPAs were used for every ground experiment so that results could be compared with similar flight data. Depending upon the ground experiment, each FPA held either one, two, or three 4 ml samples, which were separated by rubber septa.

3.2.2 Clinostat

Two clinostats were used to simulate the "indirect" effects of hypogravity on the ground. The largest clinostat, which was built at the beginning of this research, held up to eight FPAs. A motor rotated these FPAs in a horizontal position at approximately 8 revolutions/min, which resulted in a maximum centrifugal acceleration of about 5×10^{-4} *g*. Each FPA on the clinostat contained two 4 ml samples. The samples were on each end of the tube, which allowed them to be measured in the spectrophotometer. The samples were hard filled (no air bubbles) and were separated by rubber septa.

Because the direction of the gravity vector was constantly changing, the cells did not sediment to the bottom of the FPA. Instead they sedimented in a small circular motion during each rotation of the clinostat. This is shown in Figure 3-1.

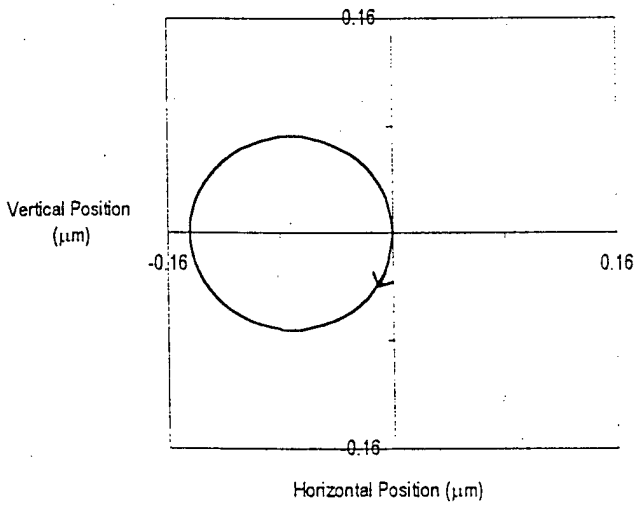

Figure 3-1 Two Dimensional Position of Bacterium During Clinorotation. The figure shows a non-rotating coordinate frame, looking at the end of a culture tube. The origin is the bacterium's original location. As the clinostat rotates counter-clockwise.

The circumference of this circle can be found by multiplying the sedimentation rate by the rotational period (see Appendix A for sedimentation calculation).

$$\text{Circumference} = V_{sed}\left(T\right) = 0.06\frac{\mu m}{sec}\left(7.5\ sec\right) = 0.45\ \mu m \tag{3-1}$$

The circle's diameter can then easily be found.

$$\text{Diameter} = \frac{0.45\mu m}{\pi} = 0.14\mu m \tag{3-2}$$

This distance is much smaller than the bacterium's expected diffusion distance. In one dimension this distance is

$$\left\langle x\right\rangle = \sqrt{2Dt} = \sqrt{2\left(0.3\frac{\mu m^2}{sec}\right)7.5\ sec} = 2.1\mu m \text{ (reference Appendix A).} \tag{3-3}$$

In two dimensions, the bacterium's expected diffusion distance is

$$\left\langle r\right\rangle = \sqrt{4Dt} = \sqrt{4\left(0.3\frac{\mu m^2}{sec}\right)7.5\ sec} = 3.0\mu m \text{ (Einstein, 1956).} \tag{3-4}$$

Because this random motion due to diffusion is much larger than the displacement due to sedimentation during clinorotation, the cells are in a state of "functional weightlessness" (Klaus *et al.*, 1997).

The other clinostat only rotated one FPA, which contained two 4 ml samples. This clinostat was inclined 30° (reference Figure 3-2). In this orientation only the component of the gravity vector that was perpendicular to the tube's axis was compensated by clinorotation. The other component, which was parallel to the tube's axis, was not affected by clinorotation. As a result, bacteria on the inclined clinostat sedimented at a rate corresponding to 0.5 *g* down the length of the FPA ($\sin(30°)=0.5$). Instead of the circular sedimentation motion associated with the

horizontal clinostat, the inclined clinostat caused bacteria to sediment in a spiraling motion. The diameter of this spiraling motion was about cos(30°)1.4 μm = 1.2 μm. and bacteria moved 0.225 μm down the length of the tube (7.5 sec × 0.06μm/sec × 0.5) during each rotation. This new use of clinorotation allowed acceleration forces between 0 *g* and 1 *g* to be simulated on the ground. Similar experiments could later be used to model the Martian or Lunar gravity environments.

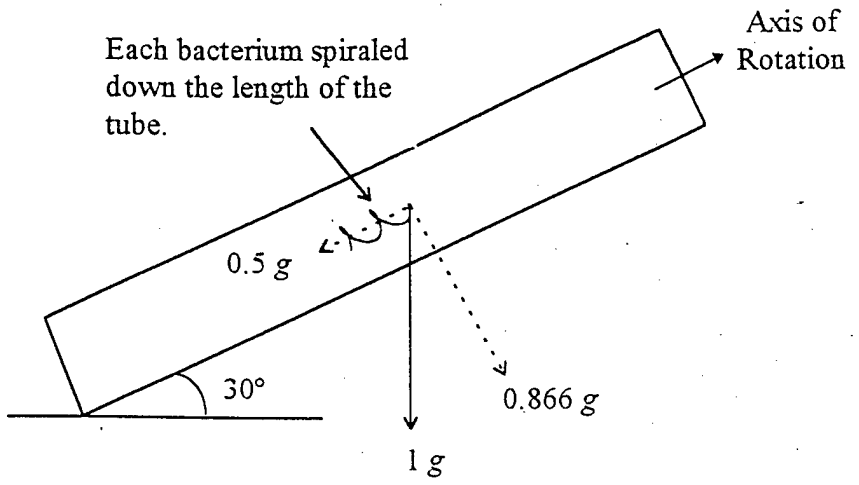

Figure 3-2 Side View of Inclined Clinostat. On a 30° inclined clinostat, the bacteria sedimented down the length of the FPA at a rate corresponding to 0.5 *g*. Clinorotation canceled any net effect of sedimentation perpendicular to the tube's axis.

3.2.3 Centrifuge

An International Equipment Corporation Model HN-S centrifuge was operated at 500, 1000, or 1500 RPM. This corresponded to acceleration forces of 50, 180, or 400 *g*, with estimated accuracies of ±10, ±40, or ±50 *g*, respectively. Because the

centrifuge held only 6 FPAs, the number of samples for each experiment was limited. To increase the *n*-value each FPA held three 4 ml samples, which were separated by rubber septa.

Because the cells sedimented very quickly in the centrifuge, they had to be shaken prior to each optical density measurement. This was accomplished by injecting air into the FPA, shaking the tube vigorously, and using a Vortex Genie. Microscopy confirmed this technique resulted in a colloidal sample, which allowed accurate optical density readings, even after accelerations of up to 700 *g*.

Due to size limitations, the middle sample in each FPA could not be measured in the spectrophotometer directly. Therefore, the centrifuged samples were transferred to 9 ml containers for measurement. After being measured, each sample was removed from the experiment.

3.3 DATA ANALYSIS

3.3.1 Correlation of Optical Density and Cell Population

Cell density was determined approximately every 5 to 12 hours using a Bausch & Lomb Spectronic 20 model spectrophotometer at 600 nm. For most of these measurements air was injected into the FPA, and the culture was vortexed prior to determining the optical density. These samples were then removed from the experiment.

There were two exceptions to this general procedure. Because samples on both clinostats did not sediment significantly, they were only shaken immediately before the last data point. In addition, the first few measurements of the 1 *g* controls were also measured without shaking. For both of these conditions, test samples were measured with and without shaking, and no difference was detected. It was therefore determined that these samples did not need to be disturbed to obtain accurate readings. Consequently, these samples were not removed from the experiment.

The correlation of OD_{600} and cell population for the FPAs was determined at the beginning of this research. This was accomplished by plotting the 32 data points shown in Figure 3-3. Using a regression analysis, the least squares line through these points was determined to be

$$\text{Cells/ml} = 2.1 \times 10^9 \text{ (FPA OD)} \div 8.7 \times 10^7.$$

This linear curve fit had an R^2 value of 0.982.

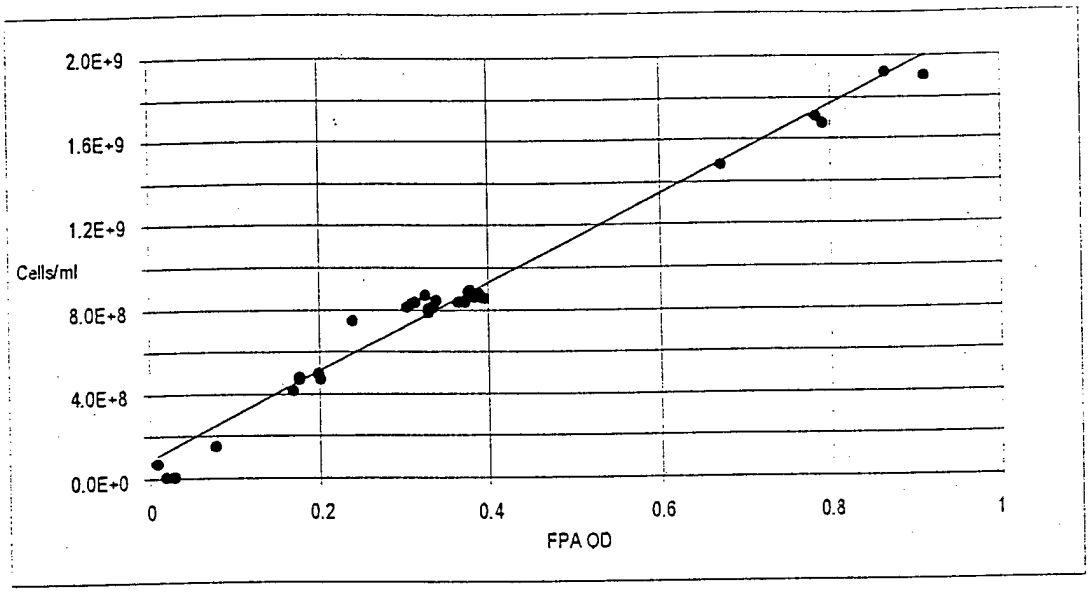

Figure 3-3 Correlation of FPA Optical Density and Cell Population. Linear regression equation was Cells/ml = 2.1×10^9 (FPA OD) + 8.7×10^7.

Because each FPA had different optical characteristics, a baseline optical density measurement was made on each FPA immediately after they were loaded. Subsequent measurements from each FPA were corrected by subtracting the baseline reading from the same FPA. This difference was used to calculate the cell density, which allowed comparison of growth in different FPAs.

Because the optical density for the centrifuge samples was measured in 9 ml culture tubes, yet the controls and clinostat experiments were measured in FPAs, it was necessary to convert each 9 ml optical density measurement to an equivalent OD_{600} reading in an FPA. This was accomplished by measuring the 32 data points shown in Figure 3-3 in an FPA and a 9 ml container. A second order curve was then fitted through these points. The resulting calibration equation was

$$\text{FPA OD} = 0.2102\ (9\text{ ml OD})^2 + 0.6054\ (9\text{ ml OD}).$$

This equation allowed centrifuge data, measured in 9 ml culture tubes, to be compared with clinostat and static samples, which were measured in FPAs. The curve, shown in Figure 3-4, had an R^2 value of 0.990.

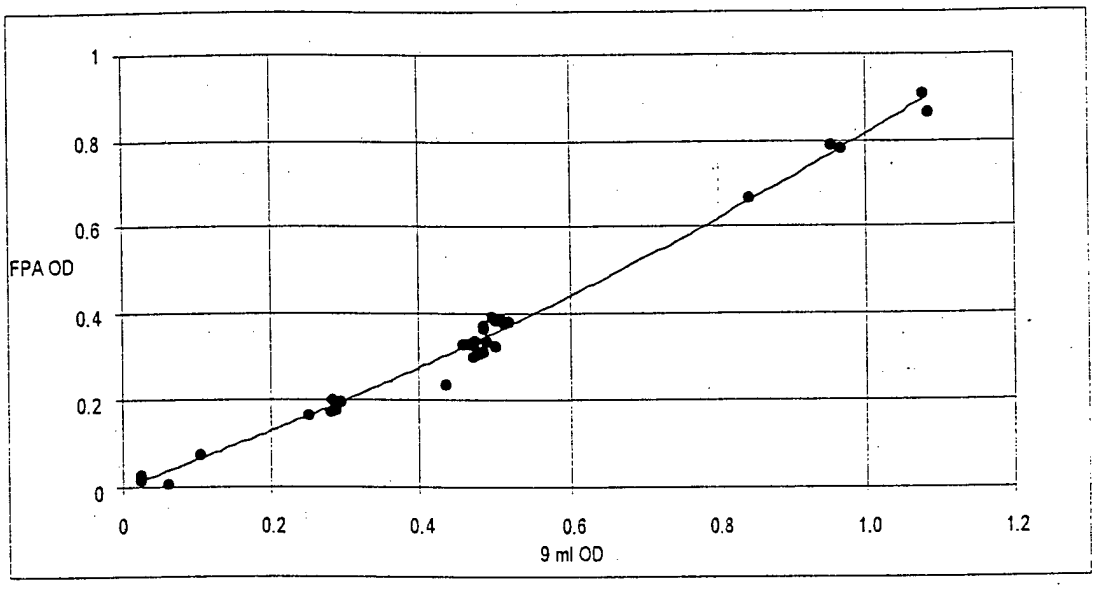

Figure 3-4 Correlation of FPA Optical Density and 9 ml Optical Density. The regression equation was FPA OD = 0.2102 (9 ml OD)^2 + 0.6054 (9 ml OD).

3.3.2 Analysis of Growth Kinetics

An objective method of analyzing the growth data was desired. This was accomplished using the bacterial growth curve shown in Equation 3-5 (Bailey and Ollis, 1986; Kacena and Todd, 1997) and a Marquardt least-squares curve fitting

algorithm (Marquardt, 1963). The curve was determined using SigmaPlot Scientific Graph System by Jandel Scientific.

$$C(t) = C_i + \frac{C_0 e^{\mu t}}{1 - \frac{C_0}{C_S}\left(1 - e^{\mu t}\right)} \tag{3-5}$$

Inputs for the algorithm were time (t) and the corresponding cell density ($C(t)$) from a number of different times. Using these inputs, the algorithm determined C_i, C_s, C_0, and μ. The algorithm required initial estimates for each of these four parameters. For consistency, and in an effort to eliminate subjective calculations of the parameters, the initial estimates were the same nominal values for every growth curve. The initial cell density was $C(t = 0) = C_i + C_0$; the final cell density was $C(t \to \infty) = C_i + C_s$; and μ was the exponential growth rate.

As an example, Figure 3-5 shows a typical growth curve from a 1 g experiment at 21°C, based upon 12 experimental data points. For this particular curve, the R^2 value for the fitted growth curve was 0.995, meaning the curve accounted for 99.5% of the variability in the data (Montgomery and Runger, 1994). This was slightly lower than the average R^2 value of 0.997.

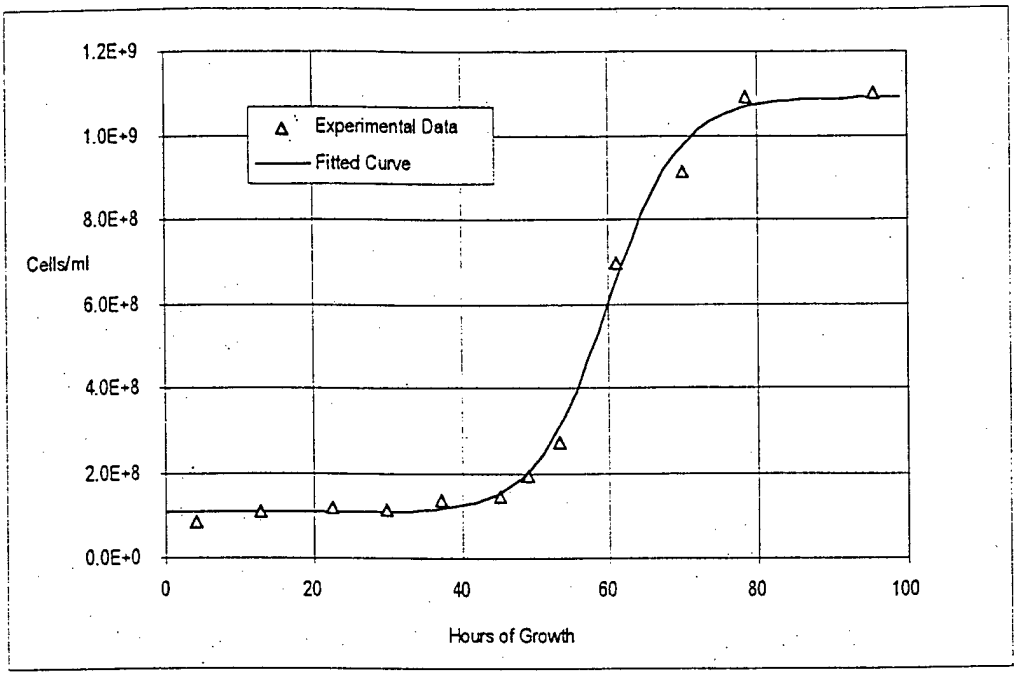

Figure 3-5 Fitted Growth Curve for *E. coli*. This experiment was conducted in 1 *g* at 21 °C. The curve was fitted using Equation 3-5.

From the four variables, C_i, C_s, C_0, and μ, four growth parameters were determined for each curve -- the length of the lag phase, the length of the exponential phase, the doubling time, and the final cell density. Final cell density was $C(t \to \infty) = C_i + C_s$. The time required for the cells to double during the exponential phase was found by Equation 3-6 (Bailey and Ollis, 1986).

$$T_{double} = \frac{\ln(2)}{\mu} \tag{3-6}$$

Determining the length of the lag and exponential phases was slightly more involved. First, two points on the exponential portion of the growth curve were found, referred to as point A and point B. The time and approximate cell concentrations for these points are shown in Equations 3-7 and 3-8.

$$t_A = \frac{1}{\mu}\ln\left(\frac{C_i}{C_0}\right) \qquad C(t_A) \approx 2C_i \tag{3-7}$$

$$t_B = \frac{1}{\mu}\ln\left(\frac{C_S}{C_0}\right) \qquad C(t_B) \approx \frac{Cs}{2} \tag{3-8}$$

Because C_0 was very small relative to C_i and C_s, the value of $C(t_A)$ was approximately twice the initial cell count (one doubling had occurred), and the value of $C(t_B)$ was about half of the final cell count (one doubling remained). Therefore, these two points were both on the exponential portion of the growth curve.

By extrapolating the line between points A and B, using the exact values for $C(t_A)$ and $C(t_B)$, the lengths of the lag and exponential phases were determined mathematically using Equations 3-9 and 3-10.

$$t_{\text{End of Lag}} = t_A - \left(\frac{t_B - t_A}{\log\left(C\left(t_B\right)\right) - \log\left(C\left(t_A\right)\right)}\right)\left(\log\left(C\left(t_A\right)\right) - \log\left(C_o + C_i\right)\right) \tag{3-9}$$

$$t_{\text{End of Exp}} = t_A - \left(\frac{t_B - t_A}{\log\left(C\left(t_B\right)\right) - \log\left(C\left(t_A\right)\right)}\right)\left(\log\left(C\left(t_A\right)\right) - \log\left(C_s + C_i\right)\right) \tag{3-10}$$

This is more clearly shown graphically in Figure 3-6, which plots the same experimental data and curve as Figure 3-5. However, Figure 3-6 uses a semi-log plot, which is a standard means of identifying when the exponential phase begins and ends (Atlas, 1997; Black, 1993; Carpenter, 1972). The point where the extrapolated line between A and B intersected the initial cell concentration defined the end of the lag phase and the beginning of the exponential phase. Likewise, the point where the extrapolated line intersected the final cell density defined the end of the exponential phase.

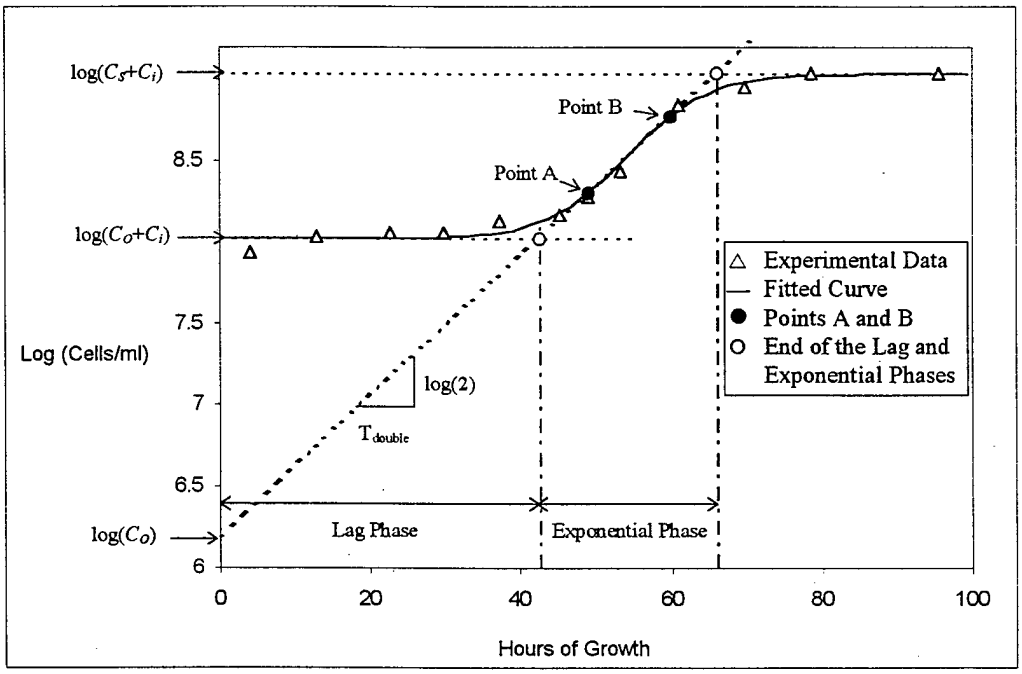

Figure 3-6 Graphical Determination of the Length of the Lag and Exponential Phases. All of the calculated parameters are shown, using the same data from Figure 3-5. This plot, however, uses a semi-log scale.

This analysis was accomplished for a number of growth curves, derived from independent data points during each experiment. The final cell density, growth rate, length of the lag phase, and length of the exponential phase for each experiment were compared with those of controls. The statistical significance of any differences between data pairs was determined using a Student's *t*-test with $p < 0.05$ and degrees of freedom determined by the number of replicate cultures.

CHAPTER 4

GRAVITY-DEPENDENT FACTORS

AFFECTING BACTERIAL GROWTH

4.1 ABSTRACT

The existing theory that sedimentation indirectly affects the lag phase of suspended bacterial cultures was investigated through experimental and analytical analyses. Experimental data supported this theory, indicating that the presence of excreted by-products shortens *E. coli's* lag phase. However, computer simulations did not completely support this theory. These simulations, modeling diffusion and sedimentation, indicated that there is no difference in the initial concentration of by-products around bacteria on orbit compared with similar experiments on Earth. It is therefore proposed that in addition to sedimentation, density-driven convection also affects bacterial growth, causing by-products that are beneficial to growth to rise toward the surface. The absence of this force on orbit may be the primary physical mechanism that can explain the shortened lag phase observed in suspended bacterial cultures on orbit.

4.2 BACKGROUND

The most plausible explanation for the observed altered bacterial growth in space is that gravity indirectly affects bacterial growth through changes in the bacteria's sedimentation rate. As explained in Chapter 2, this theory has been supported by a number of ground and space-flight experiments. It proposes that bacteria on Earth sediment away from their cofactors and enzymes. In contrast, bacteria in space remain surrounded by their enzymes, and as a result come out of lag phase sooner (Klaus *et al.*, 1997).

This explanation is based upon two assumptions. It assumes bacteria have a shorter lag phase when they are surrounded by high concentrations of their by-products. This assumption is supported with previous ground studies (Barford *et al.*, 1982). The above theory also presumes that the absence of sedimentation on orbit allows bacteria to remain surrounded by a higher concentration of excreted by-products than they do on Earth. This first assumption was tested experimentally by growing *E. coli* in preconditioned medium. The second assumption was evaluated analytically using a computer simulation. The methods and results from both of these investigations are discussed in the following sections.

4.3 EXPERIMENTAL DATA

4.3.1 Experiment and Hypothesis

Two experiments were conducted to test the assumption that *E. coli* have a shorter lag phase when surrounded by their by-products. These experiments grew *E. coli* at 1 *g* in different proportions of preconditioned medium, which was collected near the end of the lag phase. It was hypothesized that cultures grown with preconditioned medium would have a shorter lag phase than cultures grown in fresh medium.

4.3.2 Specific Materials and Methods

E. coli was grown in FPAs without air bubbles. Similar to other experiments, the inoculum of 10^6 cells/ml was taken from a saturated culture without glucose. Near the end of the anticipated lag phase the bacteria were removed using a 0.22 μm filter; this occurred 20 to 24 hours after inoculation.

The preconditioned medium from these samples was collected. Some of this medium was inoculated with 10^6 cells/ml. A mixture of 50% preconditioned medium and 50% fresh medium was also inoculated with 10^6 cells/ml. In addition, controls using 100% fresh medium were also inoculated with the same concentration of cells. These samples were then separated into 4 ml samples and cultivated anaerobically in FPAs. As detailed in Chapter 3, the cell densities were measured approximately every 4 to 8 hours, and the length of the lag phase was determined analytically. The

statistical significance of any differences between data pairs was determined using a Student's *t*-test with $p < 0.05$ and degrees of freedom determined by the number of replicate growth curves.

The first set of experiments had an n value of 5 for every culture, except those grown in 100% preconditioned medium. Because one of these samples was broken, this particular experiment only had an n value of 4. The second set of experiments, which used the exact same materials and methods, had an n value of 10 for every experiment.

Two steps were taken to confirm these experimental methods were valid. During the experiment three 4 ml samples of preconditioned medium were not inoculated and were observed for 5 days. These samples showed no signs of growth, which indicated all the bacteria were removed by the filter. To further validate the experimental methods, *E. coli* was also grown in 100% fresh medium with higher inoculations than the controls. These inoculations of 1.5×10^6 cells/ml and 2×10^6 cells/ml were expected to have a shorter lag phase than the controls, which were grown in fresh medium and inoculated at only 1×10^6 cells/ml. This would be consistent with previous research (Barford *et al.*, 1982) and allow confirmation of the analysis methods discussed in Chapter 3.

4.3.3 Results

As hypothesized, for both experiments the average lag phase was shorter when the cultures were grown in preconditioned medium. As an example, Figure 4-1 shows

the average growth curves for the second experiment. For this experiment the average lag phase using 100% preconditioned medium was 1.4 hours shorter than the controls ($p = 0.05$), and the samples cultivated in 50% preconditioned medium had a 0.3 hour shorter lag phase on average when compared with control samples ($p = 0.36$).

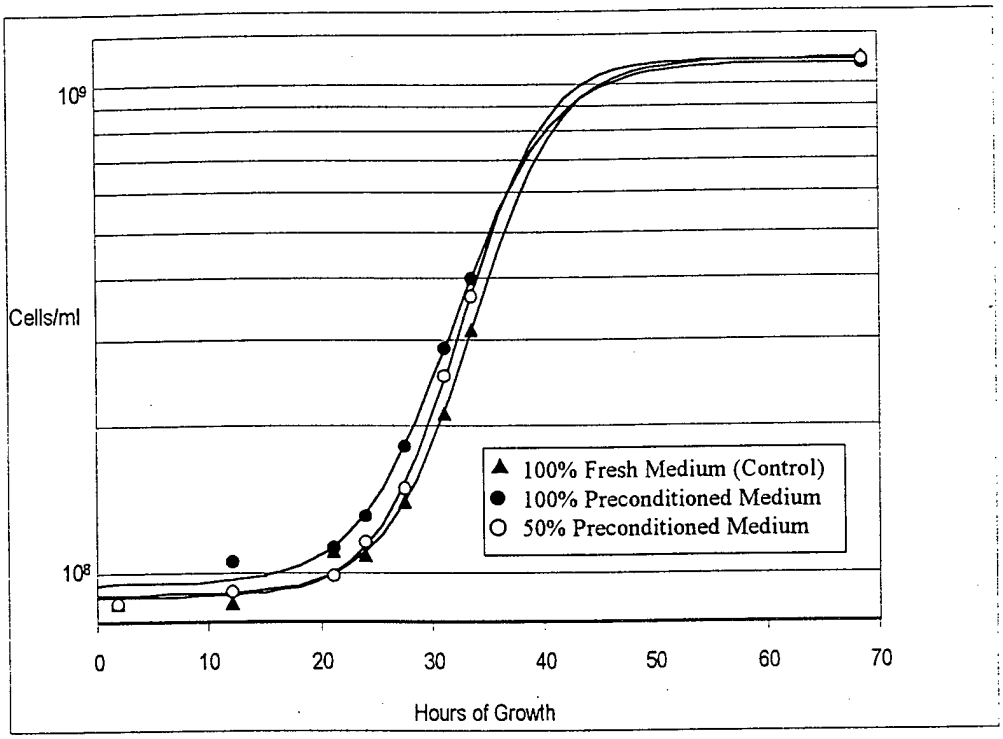

Figure 4-1 Sample Results from a Lag Phase Experiment. The figure shows the average cell densities and growth curves for different percentages of fresh and preconditioned medium. Higher concentrations of preconditioned medium resulted in shorter lag phases.

This data, along with the results from the first experiment, are summarized in Table 4-1. Notice, both sets of experiments had similar results. When the data from both experiments were combined, the lag phase for the samples grown in 100%

preconditioned medium averaged 1.3 hours shorter than the controls (p = 0.02), and the samples cultivated in 50% preconditioned medium had a 0.9 hour shorter lag phase on average (p = 0.06). These two comparisons are shaded in Table 4-1.

Table 4-1 Experimental Results of Variables Affecting Lag Phase

		Length of the Lag Phase (hours)					
		Experiment		Controls		Difference	
Experiment	n values[a]	Ave.	Std.	Ave.	Std.	Ave.	p
100% Precond. Med.:							
First	4-5	24.21	1.95	25.36	0.31	-1.1	0.14
Second	10-10	23.58	1.79	24.95	1.66	-1.4*	0.05
Total	14-15	23.76	1.78	25.08	1.36	-1.3*	0.02
50% Precond. Med:							
First	5-5	23.13	2.18	25.36	0.31	-2.2	0.08
Second	10-10	24.69	1.40	24.95	1.66	-0.3	0.36
Total	15-15	24.17	1.79	25.08	1.36	-0.9	0.06
1.5×10^6 cells/ml:							
First	5-5	23.29	0.60	25.36	0.31	-2.1*	<0.01
Second	10-10	23.21	2.02	24.95	1.66	-1.7*	0.02
Total	15-15	23.23	1.65	25.08	1.36	-1.8*	<0.01
2×10^6 cells/ml:							
First	5-5	22.00	1.51	25.36	0.31	-3.4*	<0.01
Second	10-10	23.93	0.93	24.95	1.66	-1.0	0.06
Total	15-15	23.29	1.45	25.08	1.36	-1.8*	<0.01

[a] n values show the number of experimental growth curves and control growth curves respectively.
* Indicates statistically significant difference compared with controls ($p < 0.05$).

As shown in the bottom two sections of Table 4-1, experimental results also indicated that cultures in 100% fresh medium had a shorter lag phase when they were inoculated with higher concentrations of cells. On average, experiments with initial concentrations of 1.5×10^6 or 2×10^6 cells/ml had a 1.8 shorter lag phase than the controls, which had an inoculation of 1×10^6 cells/ml. These results are consistent

with previous research (Barford *et al.*, 1982), which indicates the analytical methods used to determine the differences in the lag phase were reasonably accurate.

4.3.4 Discussion

Both sets of experiments supported the proposed hypothesis that higher concentrations of preconditioned medium result in a shorter lag phase. On average, experiments grown in 100% preconditioned medium had a 1.3 hour shorter lag phase than controls, and those grown in 50% preconditioned medium had a 0.9 hour shorter lag phase than controls. These findings suggest that if space flight results in a higher initial concentration of by-products around each bacterium, they would also have a shorter lag phase.

4.4 ANALYTICAL ANALYSIS

Analytical analysis of the gravity-dependent factors affecting bacterial growth was also accomplished. This was intended to test the theory that the absence of gravity allows bacteria to remain surrounded by relatively high concentrations of by-products. It is believed that only after cells on Earth sediment to the bottom of their culture tube are they surrounded by more bacterial waste than they would be on orbit (Klaus *et al.*, 1997). This theory is illustrated graphically in Figure 4-2.

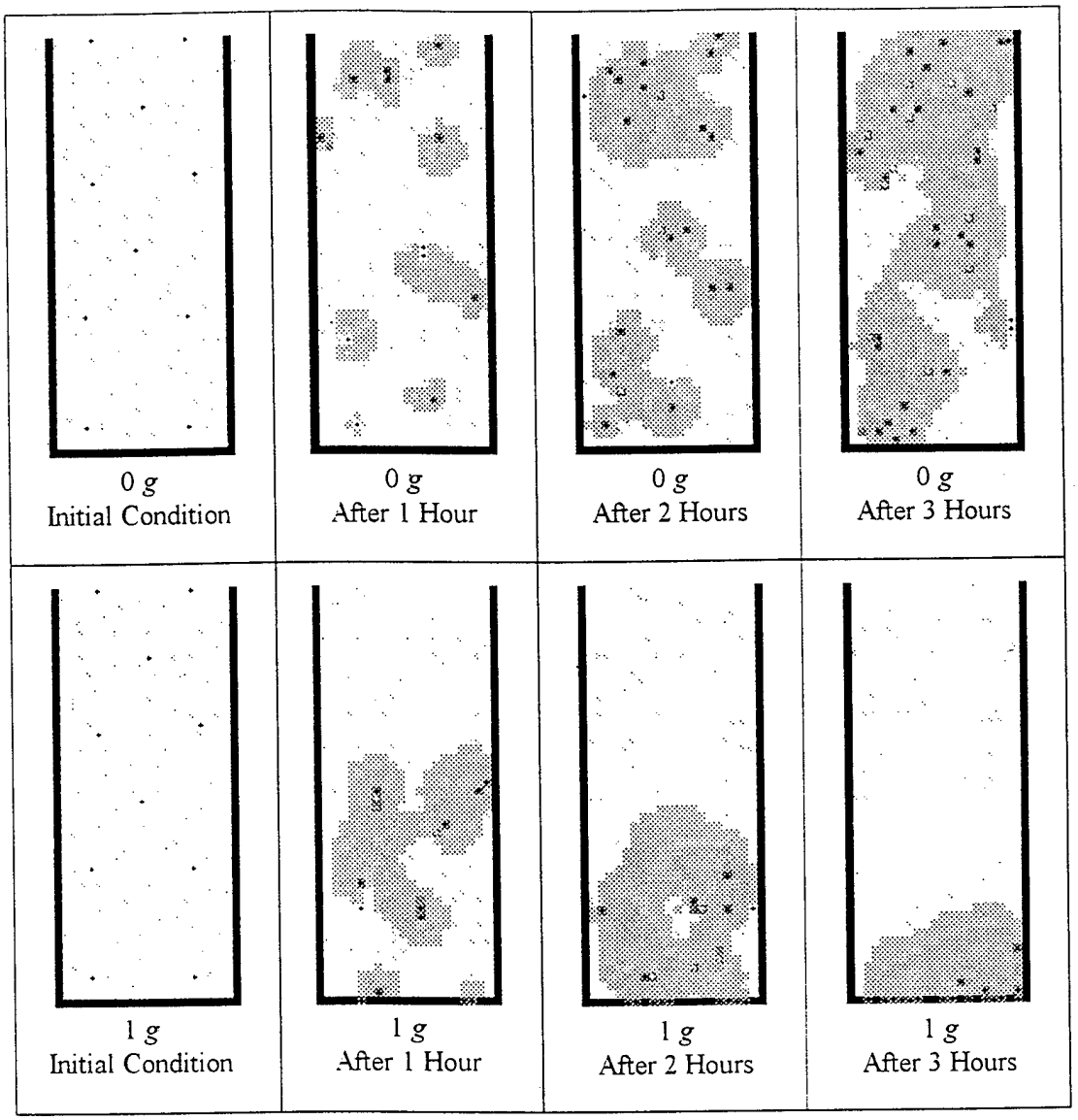

Figure 4-2 Graphical Representation of Existing Theory. The black dots in each test tube represent bacteria, the small gray dots are glucose molecules, and the "cloud" around each bacterium represents bacterial by-products. As shown above, in 1 *g* bacteria are believed to sediment away from their excreted by-products. In microgravity, however, it has been proposed that the lack of sedimentation allows bacteria to remain surrounded by their by-products, which causes a shorter lag phase. Eventually, in 1 *g* bacteria also accumulate at the bottom of their culture tube, where the lack of nutrients is believed to restrict growth (Klaus, 1998; C. Lanning, Undergraduate Research Opportunity Program, University of Colorado, Boulder, CO, USA, 1998).

4.4.1 Preliminary Analysis

The preliminary analysis of the effects of sedimentation began with the following calculations. An *E. coli* bacterium sediments at a rate of 0.06 μm/sec (see Appendix A for calculations). The concentration of its by-products released at one time can be modeled as a normal distribution with a standard deviation of $\sqrt{2Dt}$, where D is the diffusion coefficient (Einstein, 1956; Berg, 1983). Therefore, the time required for a bacterium to sediment one standard deviation away from its own by-products can be found by solving for t in the equation $0.06t = \sqrt{2Dt}$. For a typical by-product diffusion coefficient on the order of 10^{-5} to 10^{-6} cm^2/sec (Cussler, 1997), t = 15.4 to 154 hours.

This shows the effects of gravity are eventually greater than random Brownian motion. However, this result raises two questions. First, the above analysis only addressed by-products that were excreted over 15 hours in the past. What about the by-products that were released just a few minutes in the past? They should have a much greater effect on the concentration of by-products around the cells. Secondly, in the time it takes for one bacterium to fall away from its own by-products (15.4 hours to 154 hours), the cell will sediment a distance of approximately 3,300 to 33,000 μm. This is a long distance, considering bacteria with a concentration of 10^6 cells/ml have an intercellular separation of only 120 μm (see Appendix A). This means in the time required for one bacterium to fall away from its own waste, it will pass through the by-products left by approximately 27 to 270 other bacteria. Would this exposure to other cells' by-products offset any effects of sedimenting away from its own by-products?

4.4.2 Computer Simulation

These two questions were investigated using a FORTRAN computer simulation. (See Appendix A for flow-chart and code.) This simulation was designed to quantitatively determine the relative levels of by-products around bacteria in different inertial environments while considering motion due to only two forces -- sedimentation and diffusion. Unlike the computer model used to generate Figure 4-2, this simulation was not graphical. However, it did use more realistic numbers to accurately model the physical environment.

The simulation modeled diffusion and sedimentation of up to 50 bacteria, as well as diffusion of the by-products in two dimensions. Every minute during the simulation the bacteria sedimented downward and diffused a random distance in both dimensions. Each bacterium also excreted one by-product every minute. For the initial simulations this was assumed to be a large protein with a diffusion coefficient of 10^{-7} cm^2/sec (Klaus *et al.*, 1997). If these simulations indicated bacteria did sediment away from these relatively large by-products, additional simulations were planned using successfully smaller diffusion coefficients. Every 10 minutes the program calculated the expected number of by-products within a 10 μm grid of a specific bacterium. If sedimentation causes bacteria to "fall away" from their excreted by-products, the 0 *g* simulation should have a higher initial concentration of by-products near the specified bacterium than the 1 *g* simulation.

4.4.3 Simulation Results with One Bacterium

Figure 4-3 shows the simulation results with only one bacterium, which was initially located 5 mm from the bottom of the culture tube. The expected number of by-products around the bacterium was initially higher in 0 *g*, than in 1 *g*, indicating the lack of sedimentation allowed the cell to remain near its excreted by-products. Only after the cell had sedimented to the bottom of the tube in 1 *g* was the expected number of by-products around the cell greater in 1 *g* than it was in 0 *g*.

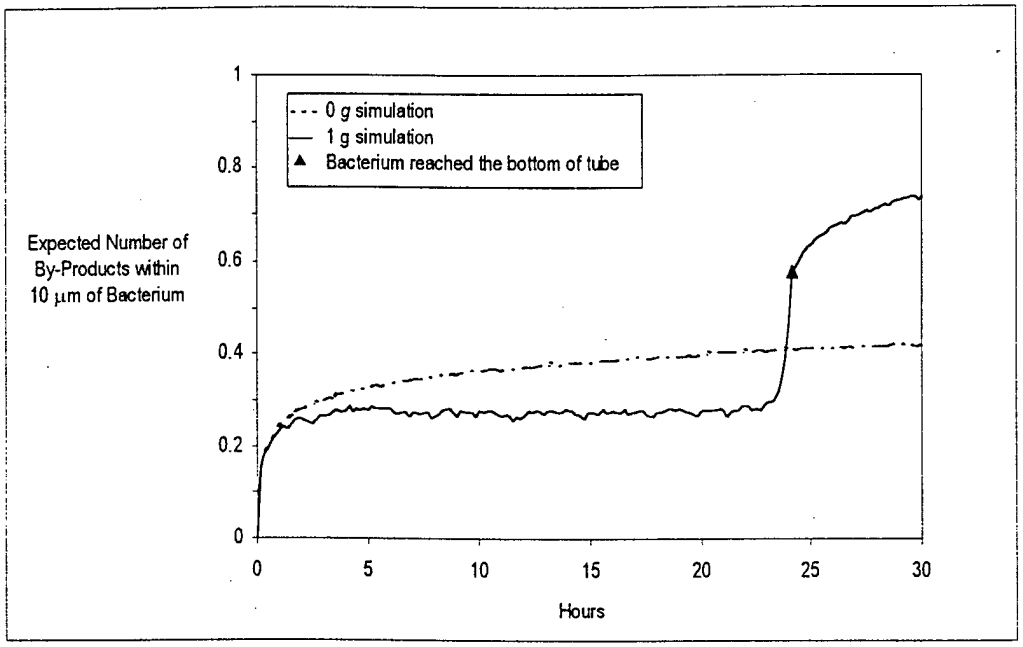

Figure 4-3 Results of Simulation with One Bacterium. In 0 *g* there were initially more by-products expected near the bacterium. However, after the cell in the 1 *g* simulation reached the bottom of the culture tube, more by-products were expected near the bacterium in 1 *g*.

This answered the first question discussed above. Although it can take a long time for a bacterium to fall farther than one standard deviation away from its by-products, sedimentation does result in a lower initial concentration of by-products around one bacterium. Considering the experimental data discussed previously, the higher concentration of by-products surrounding a bacterium in 0 *g*, should result in a shorter lag phase than similar experiments in 1 *g*. These results also show that eventually even one bacterium will become surrounded by a relatively high concentration of by-products after sedimenting to the bottom of its culture tube.

4.4.4 Simulation Results with 50 Bacteria

The second question, concerning the effects of sedimenting through the by-products left by other bacteria, was addressed by running the same simulation with 50 bacteria. The first bacterium was initially placed in the same location as the previous simulation. An additional 49 cells were then randomly placed in the two-dimensional culture tube around this bacterium. As shown in Figure 4-4, no difference was detected in the expected initial number of by-products around the first bacterium. The only difference occurred after the bacterium had sedimented close to the bottom of the tube. Other simulations, with the bacteria farther from the bottom of the culture tube showed no difference in 1 *g* or 0 *g* during the entire simulation. This indicated that because sedimentation takes a relatively long time to separate a bacterium from its by-products, the effect of one bacterium falling away from its own by-products is offset by the waste excreted by other bacteria.

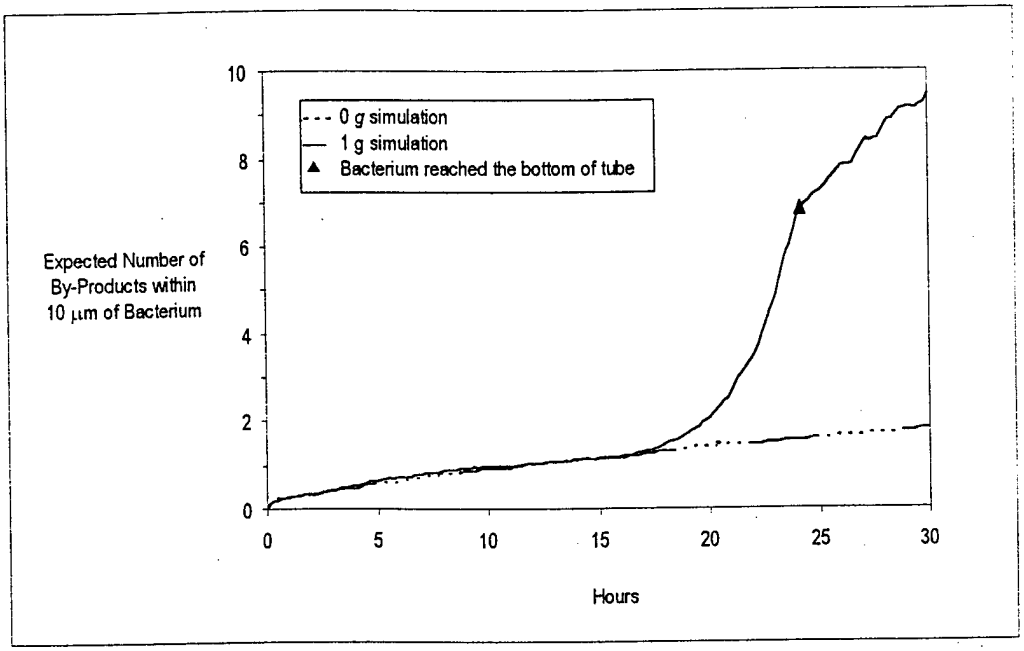

Figure 4-4 Results of Simulation with 50 Bacteria. There was no initial difference in the expected number of by-products around the first bacterium in 1 *g* and 0 *g*.

Deficiencies in the simulation program actually made the effect of sedimentation more dramatic than it should have been. For example, the simulation used a diffusion coefficient of 10^{-7} cm^2/sec for the by-products, which were assumed to be large proteins. However, this was at least an order of magnitude smaller than the coefficient for most common metabolites of *E. coli* (Cussler, 1997). As a result, the simulation underestimated the amount of by-product diffusion. The simulation was also only able to model 50 bacteria. If the two-dimensional culture tube was assumed to have a thickness of only 50 μm, the simulation would have had to include 1,000 bacteria to achieve a cell concentration of 10^6 cells/ml. Despite only using 50 bacteria,

and a diffusion coefficient that was at least 10 times too small, the by-products in the simulation still became evenly distributed very quickly.

4.4.5 Discussion

These simulations indicate that sedimentation alone can not account for changes in the by-product concentration before the bacteria reach the bottom of the tube. The effect of bacteria sedimenting away from their own by-products is off-set by the by-products excreted from other bacteria. Therefore, these simulations do not support the theory that claims the absence of sedimentation alone results in a higher initial concentration of by-products surrounding bacteria in space. Other experiments are therefore needed to identify the cause of lag phase and its observed shortening by reduced inertial acceleration.

4.5 CONCLUSIONS AND PROPOSED MODEL

Experimental results indicate that higher concentrations of excreted by-products result in a statistically significant shorter lag phase for suspended bacterial cultures. This supports the existing theory that gravity indirectly affects the lag phase through changes in the sedimentation rate. However, computer simulations of diffusion and sedimentation do not support this theory. These simulations showed no difference in the initial concentration of by-products around bacteria in 1 *g* and 0 *g* when more than one bacterium was included in the simulation. The only difference occurred after bacteria had accumulated at the bottom of the test tube during 1 *g*

simulations. This analysis suggests that a gravity-dependent process other than sedimentation is also involved with altering the fluid environment surrounding bacterial cells.

It is proposed here that density-driven convection, acting on the by-products excreted by each bacterium, is the dominant force that separates by-products from bacteria. This same force, acting on medium immediately surrounding crystals, is the accepted physical mechanism that explains why crystals grow to a higher level in space (Brailovskaya *et al.*, 1994; Fehribach and Rosenberger, 1989; McCay and McCay, 1994; Pusey and Naumann, 1986; Pusey *et al.*, 1988, 1986). As a crystal grows in solution, the concentration of solute immediately surrounding the crystal is reduced. This decreases the density of the surrounding medium relative to the bulk fluid. In 1 *g*, this density difference creates convection currents that rapidly rise toward the surface. However, there are no convection currents in microgravity, which allows crystals to experience larger, more uniform growth on orbit. If this same force, occurs with growing bacteria, it could explain the reports of shorter lag phase in space.

It is believed that the density difference associated with bacterial growth is caused by the consumption of nutrients from the medium immediately around each bacterium. Some of this is metabolized into various by-products which are released back into the medium. However, some of the nutrients are used for cell growth and maintenance. Therefore, the total mass of the excreted by-products is less than the mass of the glucose consumed. As a result, the medium immediately surrounding each

cell is less dense than the surrounding bulk fluid.[1] It is postulated that in a 1 g environment, this density difference creates convection currents. These currents are believed to separate by-products from their cells at a rate significantly greater than the sedimentation of *E. coli*. This is depicted graphically in Figure 4-5.

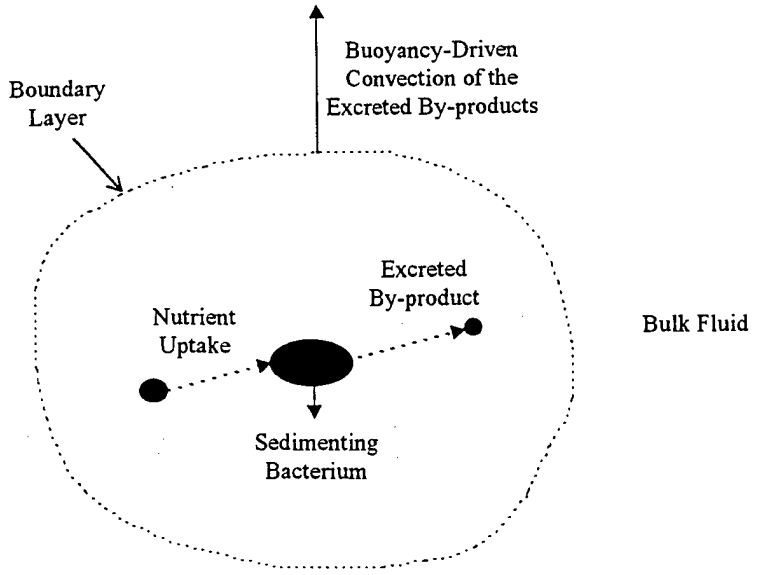

Figure 4-5 Graphical Representation of Proposed Model. As a bacterium consumes nutrients and excretes by-products, the medium immediately surrounding the cell becomes less dense than the bulk fluid. This creates buoyancy-driven convection of the excreted by-products.

Some of these by-products are believed to be beneficial for growth. Ground studies have shown that during the lag phase cells must build up the extracellular concentrations of a whole range of metabolic intermediates, particularly those of low

[1] Bacteria also produce a slight amount of heat which would further reduce the density of the medium around each bacterium. However, this is probably insignificant because heat diffuses so quickly. The diffusion coefficient for heat is approximately 1,000 times larger than the diffusion coefficient for most by-products excreted by *E. coli* (Cussler, 1997; Reynolds and Perkins, 1977). This comparison is discussed in greater detail in Chapter 6.

molecular weight, which readily diffuse across the plasma membrane (Barford *et al.*, 1992). After the concentration of these by-products reaches a critical level, the cells come out of their lag phase.

Carbon dioxide has been shown to be one of these critical by-products. A lack of dissolved carbon dioxide is a major cause of the lag phase for some bacteria (Barford *et al.*, 1992). This is possibly because carbon dioxide is required for some anaplerotic sequences, which produce intermediates of the tricarboxylic acid cycle, serving as precursors for several cellular constituents. (Gottschalk, 1986). As shown in Figure 4-6, when grown aerobically *E. coli* produce carbon dioxide, which is excreted by the cell. This is later required to produce oxaloacetate, which is an intermediate of the tricarboxylic acid cycle, and is crucial for cellular growth.

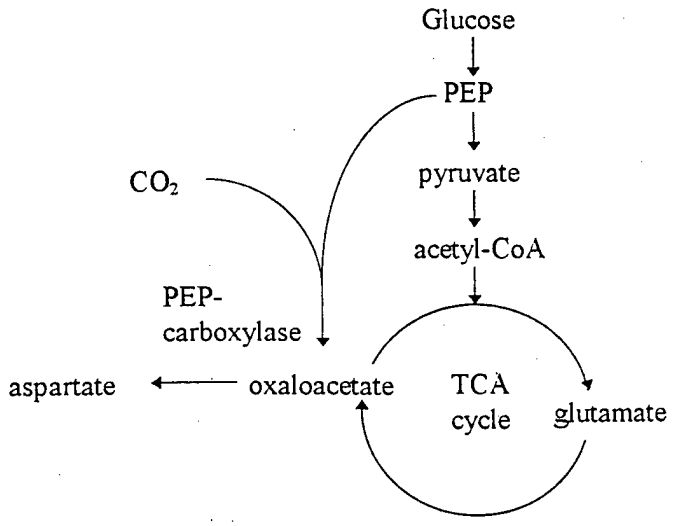

Figure 4-6 The Role of Carbon Dioxide in the Growth of *E. coli*. Dissolved carbon dioxide, which is released by other metabolic pathways, is required to produce oxaloacetate. This is an intermediate of the tricarboxylic acid cycle, which serves as a precursor for several cellular constituents (Gottschalk, 1986).

It is proposed that in a microgravity environment, the lack of convection allows beneficial by-products to remain near the bacteria. These by-products may include dissolved CO_2 (Thévenet *et al.*, 1996), particularly during the lag phase when dissolved oxygen is still present in the growth medium. However, once the exponential phase begins almost all of the dissolved oxygen is depleted from the medium (E. Smith, University of Colorado, Boulder, CO, USA, 1998), and the cells can not continue producing CO_2.

Higher concentrations of these beneficial by-products (such as CO_2 and possibly others) around the cells on orbit stimulates bacterial growth. This is believed to result in a shorter lag phase and higher final cell density than experiments on Earth. In contrast, cultures grown in 1 *g*, have small convection currents resulting from the different solute concentrations. This causes beneficial by-products to quickly rise away from the bacteria. As a result, growth is limited, and the bacteria have a longer lag phase and lower final cell density than experiments on orbit.

Eventually, in a 1 *g* environment bacteria also cluster together at the bottom of their culture tube. It is believed that this restricts their nutrient availability, which further reduces the final cell density compared with bacteria cultivated in microgravity. For example, due to sedimentation bacteria can become "stacked" on top of each other about 20 to 40 cells high (see Equations 5-2 and 6-2). In this environment, it is doubtful that bacteria at the very bottom would be able to consume any glucose. Experiments in space, however, remain colloidal, which could help explain why cultures on orbit have a higher final cell density than similar experiments on Earth.

CHAPTER 5

CLINOSTAT AND CENTRIFUGE GROWTH KINETICS OF *E. coli*

5.1 ABSTRACT

Nineteen experiments were conducted to test the proposed theory that inertial acceleration indirectly affects bacterial growth through changes in the sedimentation rate acting on the bacteria and buoyancy-driven convection acting on their excreted by-products. These experiments simulated accelerations of 0 *g* and 0.5 *g* using a clinostat, and achieved 50 *g*, 180 *g*, and 400 *g* using a centrifuge. All of the results supported the proposed model. Data consistently indicated that final cell density is inversely related to the level of acceleration. On average, clinostat experiments simulating 0 *g* produced a 16% higher final cell density than found in 1 *g* controls ($p < 0.01$), and accelerations of 400 *g* averaged a 39% lower final cell concentration than found in controls ($p < 0.01$). The effect of inertial acceleration on the lag phase was non-monotonic. Cultures under simulated 0 *g* averaged a 4.2 hour shorter lag phase than controls ($p < 0.01$), while accelerations of 50 *g* resulted in a 14.1 hour shorter lag phase than 1 *g* controls ($p < 0.01$). However, the average lag phase for *E. coli* subjected to accelerations of 400 *g* was only 0.8 hours shorter than that of

controls (p = 0.20). All of these results were consistent with trends predicted by the proposed physical model that explains how acceleration affects bacterial growth through changes in the sedimentation rate and buoyancy-driven convection.

5.2 BACKGROUND

As detailed in Chapter 2, with a few exceptions (Bouloc and D'Ari, 1991; Gasset *et al.*, 1994), most findings indicate that reduced gravity stimulates growth of suspended bacterial cultures. These experiments have consistently shown space flight results in a shorter lag phase by approximately 4 to 8 hours (Klaus *et al.*, 1994, 1997; Manko *et al.*, 1987; Mennigmann and Lange, 1986; Thévenet *et al.*, 1996) and almost twice as high a final cell density as comparable ground controls (Ciferri *et al.*, 1986; Klaus *et al.*, 1994, 1997; Kordium *et al.*, 1980; Mattoni, 1968; Mennigmann and Lange, 1986).

Clinostat experiments, which simulate the indirect effects of reduced gravity, have produced similar results to those found in space (Klaus *et al.*, 1998b; Mattoni, 1968; Mennigmann, 1994). These experiments resulted in a shorter lag phase, on the order of five hours, and approximately double the final cell density relative to controls (Klaus *et al.*, 1998b).

There have also been three reports of bacterial growth during centrifugation. In one investigation, accelerations of 2, 5, or 10 g did not affect the final cell density of suspended *E. coli* cultures (Lapchine *et al.*, 1990). This finding was supported by another investigation, in which accelerations of 3 g or 5 g did not affect *E. coli's* final

cell density (Bouloc and D'Ari, 1991). The third report involved experiments with *E. coli* at 1,000 *g* and 110,000 *g*. These accelerations resulted in an equivalent or slightly longer lag phase and a significantly lower final cell density than in 1 *g* controls (Montgomery *et al.*, 1963).

All of these results were reviewed and discussed in greater detail in Chapter 2, and their results are summarized in Table 2-3. It is important to point out that in all of the previous hypogravity experiments accelerations were close to 0 *g* (or simulated 0 *g*), and the hypergravity experiments were performed at very low or very high accelerations. An extensive literature review revealed that no one has investigated bacterial growth between approximately 0 *g* and 1 *g*, or between 10 *g* and 1,000 *g*. More importantly, no one has identified one physical mechanism that can explain all of these findings.

5.3 HYPOTHESES

As explained in Chapter 4, this research proposes that two gravity-dependent forces indirectly affect bacterial growth. In a 1 *g* environment sedimentation causes the bacterial cells to slowly accumulate at the bottom of their culture tube. It is also believed that buoyancy-driven convection causes the bacterial by-products to rise to the top of the culture tube. It is postulated that the reduced effect of convection in real or simulated hypogravity allows a relatively high concentration of excreted by-products to remain near the bacteria. This is believed to stimulate growth, resulting in a shorter lag phase. It is also postulated that this contributes to a higher final cell

density relative to 1 *g* controls. Cells on orbit (or a clinostat) also remain colloidal, which improves their nutrient availability. This is believed to also contribute to the observed higher final cell density for cultures on orbit or on a clinostat.

Based upon the proposed model, hypergravity experiments should have very different results. As acceleration increases the final cell density is expected to decrease. It is postulated that this is caused by sedimentation of the cells and buoyancy-driven convection of their by-products. Sedimentation causes the bacteria to become stacked on top of each other at the bottom of the culture tube. This is believed to limit their nutrient availability, resulting in a lower final cell density as acceleration increases. Also, as acceleration increases the buoyancy forces that act on by-products increase. Because some of these by-products are believed to be beneficial for growth, it is proposed that this also contributes to a lower final cell density as acceleration increases.

In contrast, the relationship between acceleration and the length of the lag phase is expected to non-monotonic for accelerations above 1 *g*. It is proposed that above approximately 10 *g*, sedimentation quickly causes bacteria to accumulate at the bottom of their culture tube. Before they begin doubling and become stacked on top of each other, this is beneficial for growth because the cells are surrounded by the excreted by-products from many bacteria. Because some of these by-products are initially beneficial, as shown in Chapter 4, this should result in a much shorter lag phase than in 1 *g* or 0 *g* experiments. However, as the acceleration level increases above approximately 100 *g*, buoyancy-driven convection also increases, causing the

excreted by-products to quickly move away from the bacteria. This should cause the length of the lag phase to increase, eventually resulting in no difference relative to 1 *g* controls.

These hypotheses have been divided into four separate statements, which are summarized below in italics and are shown graphically in Figures 5-1 and 5-2. The data supporting each of these hypotheses are presented in Section 5.5, and their rationales are explained further in Section 5.6.

1. Effect of Acceleration on Final Cell Density (shown graphically in Figure 5-1)

   Final cell density is inversely related to the level of acceleration (including real and simulated hypogravity).

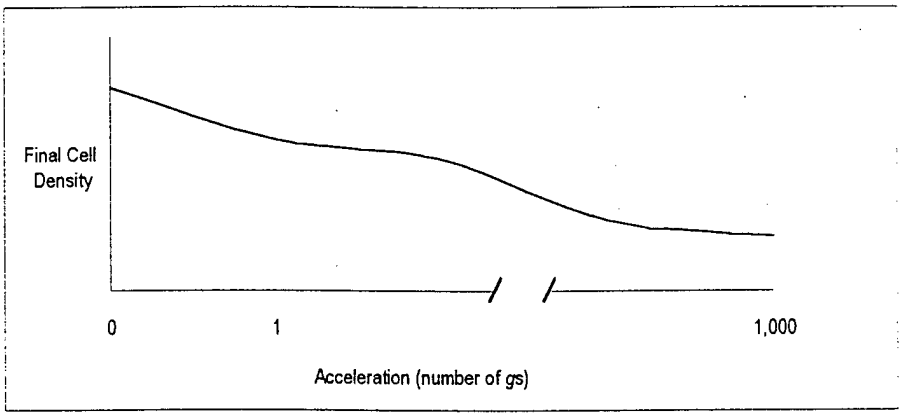

Figure 5-1 Hypothesized Effect of Acceleration on Final Cell Density

2. Effect of Acceleration on the Lag Phase (shown graphically in Figure 5-2)

   a. *For accelerations at and below 1 g (real or simulated), the length of the lag phase is directly related to the level of acceleration.*

   b. *Accelerations between approximately 10 g and 100 g result in a shorter lag phase than 1 g controls or experiments in hypogravity.*

   c. *As the acceleration increases from approximately 100 g to 1,000 g, the lag phase increases, eventually resulting in no difference relative to 1 g controls.*

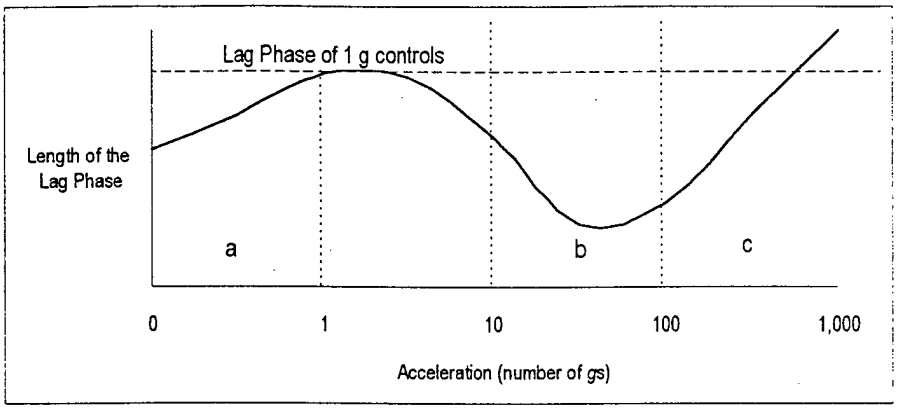

Figure 5-2 Hypothesized Effect of Acceleration on the Length of the Lag Phase. To include all three hypotheses about the lag phase on one plot, the abscissa is a log scale for accelerations greater than 1 *g*. The regions labeled a, b, and c correspond to the hypotheses listed above.

5.4 MATERIALS AND METHODS

All of the experiments discussed in this chapter used the general materials and methods outlined in Chapter 3. A horizontal clinostat was used to simulate 0 *g*; an inclined clinostat was used to simulate 0.5 *g*; and a centrifuge was used for all hypergravity experiments. Each growth curve was analyzed using the general methods presented in Chapter 3 to determine the length of the lag phase, the length of the

exponential phase, the growth rate, and the final cell density. These values were compared with controls and analyzed for statistical significance using a Student's *t*-test, with $p < 0.05$.

5.5 RESULTS

The 19 ground experiments supported all of these hypotheses. As an example, Figure 5-3 shows the average growth curves from three experiments, which were conducted at the same time. The 50 *g* samples had the shortest lag phase and the lowest final cell density. The cultures on the horizontal clinostat, simulating 0 *g*, had a higher final cell density and a shorter lag phase than the 1 *g* controls. Also, the average growth curve for the samples on the inclined clinostat, simulating 0.5 *g*, was between the 0 *g* and 1 *g* curves.

These results were typical of the rest of the experiments. Table 5-1 summarizes the results from all 19 experiments. The far left column shows the experiment numbers in chronological order and a letter to distinguish different treatment groups in the same experiment. For example, the 0 *g*, 0.5 *g*, and 50 *g* experiments shown in Figure 5-3 were all conducted at the same time and are numbered 3a, 3b, and 3c respectively in Table 5-1. The second column shows the *n* values (number of independent growth curves) for each experiment. The remaining columns compare the length of the lag phase, the length of the exponential phase, the doubling time, and the final cell density of each experiment with those of the 1 *g* controls.

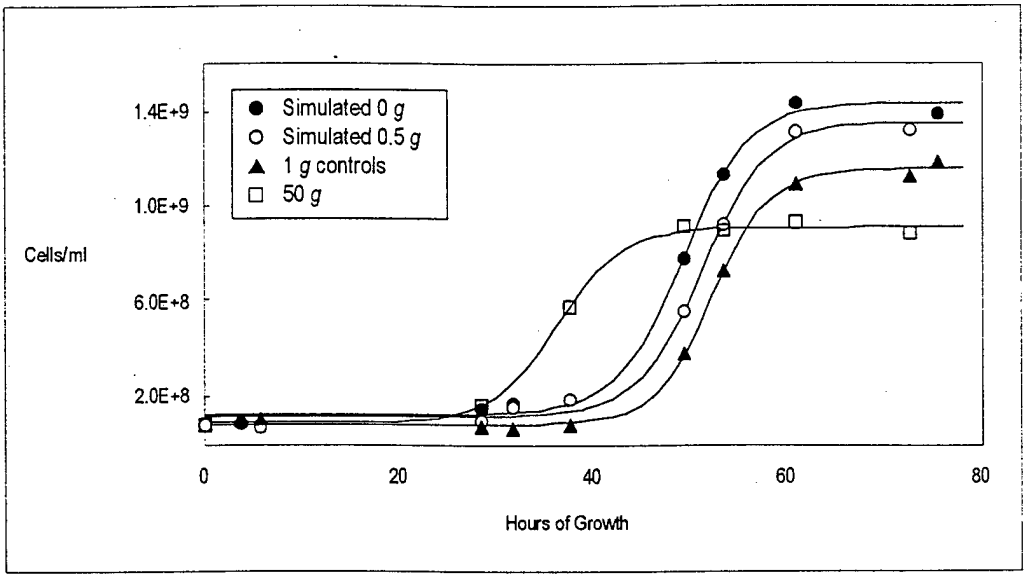

Figure 5-3 Sample of Growth Curves at Different Accelerations. The figure shows the average cell densities and average growth curves at different levels of acceleration. "Simulated" 0 *g* was achieved with a horizontal clinostat, and 0.5 *g* was "simulated" using an inclined clinostat.

The most consistent differences in the growth parameters relative to controls were the length of the lag phase and the final cell density. These are shown in the first and last vertical sections of Table 5-1, and the totals for each acceleration level are shaded. Except for the lag phase for samples at 400 *g*, all of the experiments at the same inertial condition had similar differences in their lag phase and final cell density relative to controls, and almost all of these differences were statistically significant.

Table 5-1 Summary of Growth Experiments. Totals from the most consistent results are shaded for emphasis.

		Length of the Lag Phase (hours)							Length of the Exponential Phase (hours)							Doubling Time (hours)							Final Cell Density (cells/ml x 10^8)						
		Experiment		Control		Difference			Experiment		Control		Difference			Experiment		Control		Difference			Experiment		Control		Difference		
Exp[a]	n[b]	Ave.	Std.	Ave.	Std.	Ave.	Std.[c]	p	Ave.	Std.	Ave.	Std.	Ave.	Std.[c]	p	Ave.	Std.	Ave.	Std.	Ave.	Std.[c]	p	Ave.	Std.	Ave.	Std.	Ave%	Std.[c]	p
0 g:																													
1a	6-4	35.3	3.2	36.6	1.4	-1.3	1.5	0.20	16.9	3.0	19.1	2.2	-2.1	1.6	0.11	2.44	0.39	2.81	0.29	-0.37	0.21	0.06	10.8	1.3	8.8	0.5	24%*	6%	<0.01
2a	8-6	36.9	1.8	41.1	1.1	-4.2*	0.8	<0.01	16.5	1.8	14.8	2.0	1.7	1.0	0.07	2.35	0.30	2.07	0.27	0.29*	0.15	0.04	14.3	1.4	11.6	0.4	23%*	4%	<0.01
3a	8-6	39.5	0.9	41.9	1.1	-2.4*	0.5	<0.01	23.4	1.0	25.3	1.5	-1.8*	0.7	0.01	3.33	0.12	3.63	0.21	-0.30*	0.09	<0.01	12.5	0.4	11.4	0.4	10%*	2%	<0.01
4a	8-8	36.9	2.2	41.4	1.4	-4.5*	0.9	<0.01	22.0	0.8	19.8	3.1	2.2*	1.1	0.04	3.10	0.08	2.81	0.45	0.29*	0.16	0.05	13.5	0.5	11.9	0.2	14%*	2%	<0.01
5a	3-3	54.5	3.7	60.1	1.3	-5.6*	2.3	0.02	15.5	4.7	12.3	3.2	3.2	3.3	0.19	2.30	0.68	1.84	0.48	0.46	0.48	0.20	8.5	0.1	7.6	0.6	12%*	4%	0.03
9a	6-3	31.8	4.5	35.6	1.5	-3.8*	2.0	0.05	19.6	6.8	15.2	4.0	4.4	3.6	0.13	2.76	0.93	2.17	0.57	0.59	0.50	0.14	13.0	0.6	11.5	0.7	13%*	4%	<0.01
10a	2-2	27.8	1.5	34.7	0.0	-6.9*	1.1	0.01	23.8	1.6	15.6	0.3	8.2*	1.1	<0.01	3.25	0.25	2.23	0.05	1.02*	0.18	0.02	14.0	0.5	12.1	0.6	16%*	5%	0.04
Total	41-32	37.3	6.2	41.5	6.7	-4.2*	1.5	<0.01	19.7	4.3	18.6	4.8	1.1	1.1	0.14	2.80	0.58	2.66	0.69	0.14	0.15	0.18	12.7	1.8	10.9	1.5	16%*	4%	<0.01
0.5 g:																													
1b	2-4	35.0	1.0	36.6	1.4	-1.6	1.0	0.09	17.4	0.6	19.1	2.2	-1.7	1.2	0.11	2.52	0.03	2.81	0.29	-0.29	0.15	0.06	10.3	1.6	8.8	0.5	17%	13%	0.13
2b	2-6	38.6	2.3	41.1	1.1	-2.4	1.7	0.10	16.7	2.2	14.8	2.0	1.9	1.8	0.16	2.38	0.33	2.07	0.27	0.32	0.26	0.13	13.5	0.7	11.6	0.4	17%*	5%	<0.01
3b	2-6	41.0	1.1	41.9	1.1	-1.0	0.9	0.16	24.3	0.7	25.3	1.5	-1.0	0.8	0.12	3.50	0.03	3.63	0.21	-0.14	0.09	0.08	11.6	0.9	11.4	0.4	1%	6%	0.40
4b	2-8	39.3	0.4	41.4	1.4	-2.1*	0.6	<0.01	22.5	1.3	19.8	3.1	2.8*	1.4	0.05	3.21	0.18	2.81	0.45	0.39*	0.20	0.04	13.1	0.7	11.9	0.2	10%*	4%	0.02
Total	8-24	38.5	2.5	40.5	2.2	-2.0*	1.0	0.03	20.2	3.6	20.0	4.4	0.2	1.6	0.44	2.90	0.52	2.86	0.64	0.04	0.22	0.43	12.1	1.6	11.2	1.2	8%	5%	0.07
50 g:																													
2c	3-6	25.0	1.2	41.1	1.1	-16.1*	0.8	<0.01	16.8	1.9	14.8	2.0	2.0	1.4	0.10	2.44	0.28	2.07	0.27	0.37*	0.20	0.05	9.0	0.2	11.6	0.4	-22%*	2%	<0.01
3c	3-6	24.8	6.4	41.9	1.1	-17.1*	3.7	<0.01	19.0	10.1	25.3	1.5	-6.3	5.8	0.16	2.78	1.42	3.63	0.21	-0.85	0.82	0.16	8.3	0.3	11.4	0.4	-27%*	2%	<0.01
4c	3-8	26.7	1.1	41.4	1.4	-14.7*	0.8	<0.01	17.6	1.1	19.8	3.1	-2.2	1.3	0.06	2.54	0.15	2.81	0.45	-0.27	0.18	0.08	9.2	0.4	11.9	0.2	-23%*	2%	<0.01
9b	2-3	28.8	0.9	35.6	1.5	-6.8*	1.1	<0.01	25.1	2.0	15.2	4.0	9.9*	2.7	0.02	3.83	0.29	2.17	0.57	1.66*	0.39	0.01	7.8	0.2	11.5	0.7	-31%*	3%	<0.01
10b	2-2	24.7	0.3	34.7	0.0	-10.0*	0.2	<0.01	16.7	0.9	15.6	0.3	1.1	0.6	0.12	2.53	0.15	2.23	0.05	0.30	0.11	0.06	7.2	0.0	12.1	0.6	-41%*	4%	<0.01
Total	13-25	25.9	3.1	40.0	2.8	-14.1*	1.0	<0.01	18.8	5.2	19.2	4.8	-0.5	1.7	0.39	2.77	0.77	2.74	0.70	0.03	0.26	0.45	8.4	0.8	11.7	0.4	28%*	2%	<0.01
180 g:																													
8a	3-8	16.4	4.2	24.9	1.8	-8.0*	2.5	<0.01	23.5	5.4	13.7	2.3	9.1*	3.2	0.01	3.56	0.82	1.95	0.30	1.52*	0.48	<0.01	7.5	0.3	11.7	0.5	36%*	2%	<0.01
400 g:																													
6a	3-5	33.8	0.3	32.7	3.0	1.0	1.3	0.24	18.2	0.2	19.4	3.7	-1.2	1.6	0.26	2.79	0.04	2.71	0.49	0.09	0.22	0.36	6.8	0.6	11.3	0.4	-39%*	3%	<0.01
7a	3-5	31.6	0.8	34.2	0.7	-2.6*	0.5	<0.01	14.6	2.2	14.8	1.2	-0.2	1.4	0.46	2.24	0.33	2.08	0.16	0.16	0.20	0.23	7.0	0.0	11.4	0.6	-39%*	2%	<0.01
Total	6-10	32.7	1.3	33.5	2.2	-0.8	0.9	0.20	16.4	2.4	17.1	3.6	-0.6	1.5	0.34	2.52	0.37	2.39	0.47	0.12	0.21	0.28	6.9	0.4	11.3	0.5	-39%*	2%	<0.01

* Indicates statistically significant difference compared with controls ($p < 0.05$).

[a] Numbers indicate the chronological order of each experiment. Letters are used to distinguish among different treatment groups that were initiated at the at the same time with identical controls.

[b] n values show the number of independent growth curves for the experiment and controls respectively.

[c] Standard deviations of the difference between each experimental and control parameter are a pooled estimate calculated from $\sqrt{s_1^2/n_1 + s_2^2/n_2}$. This formula was also used to estimate the standard deviation of the "Total" number of experiments for each acceleration.

The total values for change in final cell density and length of the lag phase relative to 1 *g* controls are summarized in Figures 5-4 and 5-5. Both figures use a semi-log scale for accelerations above 1 *g*, so all the data could be included on one plot. (The data for accelerations between 1 *g* and 1,000 *g* is later shown in Figures 6-7 and 6-8, without using a semi-log scale.) The figures also show one standard deviation bars for the new experimental data as well as average values from previous experiments. The new data generally agreed with previous findings. Slight differences were most likely due to different temperatures or unique experimental methods (Klaus *et al.*, 1998b; Montgomery *et al.*, 1963).

These two figures are very similar to the hypothesized results shown in Figures 5-1 and 5-2. As expected, the final cell density was inversely related to acceleration, and the length of the lag phase varied in the non-monotonic manner that was predicted.

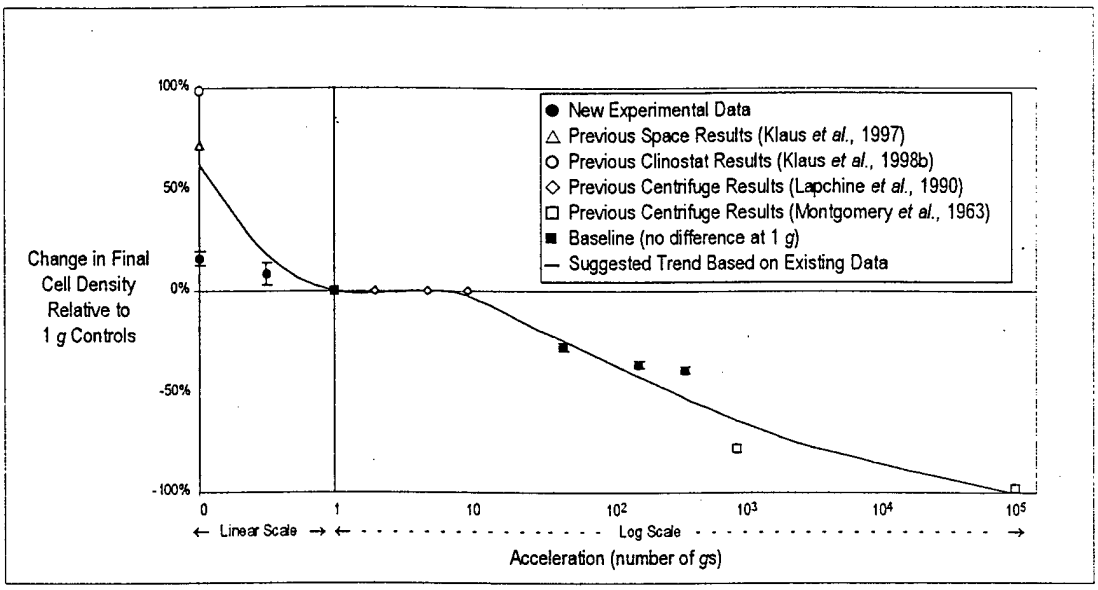

Figure 5-4 Effect of Acceleration on the Final Cell Density. To include all the data on one plot, the figure uses a semi-log scale for accelerations greater than 1 *g*.

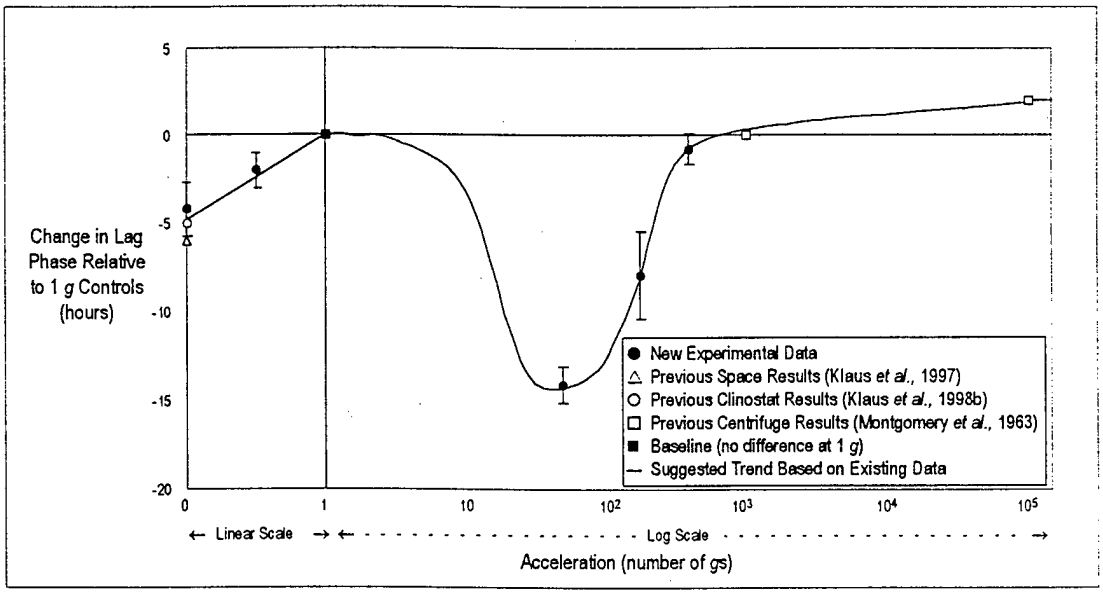

Figure 5-5 Effect of Acceleration on the Length of the Lag Phase. To include all the data on one plot, the figure uses a semi-log scale for accelerations greater than 1 *g*.

Although the effect of acceleration on the length of the exponential phase and the doubling time was not hypothesized, the results are still worthy of discussion. Unfortunately, the data for these two parameters were not as consistent as the results for the length of the lag phase or the final cell density. These inconsistent results were most likely due to only collecting a few data points during the exponential phase. Even small errors in these points could have led to inaccurate measurement of the growth rate and the length of the exponential phase. However, when all the experimental results were averaged from the 0 g simulations, the data did show some statistical trends. On average the 0 g simulations had a 1.1 hour longer exponential phase and a slightly slower growth rate (longer doubling time) than 1 g controls ($p = 0.14$ and $p = 0.18$, respectively). The 0.5 g simulations, also produced a longer average exponential phase and a slower growth rate than the control cultures, but the differences were not as dramatic. These effects of acceleration on the exponential phase were interesting because one of the largest set of space experiments had similar results. Klaus *et al.* (1997) concluded that the exponential phase was longer and the rate of growth was "unaffected (or slightly slower)" on orbit compared with similar ground controls.

5.6 DISCUSSION

The 19 ground experiments outlined in this chapter supported all four hypotheses. These hypotheses, concerning the effects of acceleration on the final cell density and the length of the lag phase, were based upon the proposed model related

to bacterial growth. This model argues that acceleration indirectly affects bacterial growth through changes in the bulk fluid environment surrounding the bacteria. It is postulated that this occurs through changes in the cells' sedimentation rate and buoyancy-driven convection acting on the bacterial by-products.

It is believed that these gravity-dependent forces caused the observed inverse relationship between acceleration and final cell density. At high acceleration levels, all the bacteria quickly became concentrated at the bottom of the culture tube. It is hypothesized that limited the nutrients available to some cells. This became a significant factor after the cells became so concentrated they were actually "stacked" on top of each other. As shown below, this condition occurred after the cell density reached only 3.1×10^{7} cells/ml, which was very early during the exponential phase.

$$\text{Cell Density when Layering Begins} = \frac{\text{Area of Septa}}{\text{Volume}\left(\pi R^{2}_{\text{Bacterium}}\right)} = \frac{1.5\times10^{8}\,\mu\text{m}^{2}}{\left(4\text{ ml}\right)\left(0.62\,\mu\text{m}\right)^{2}\pi} = 3.1\times10^{7}\,\text{cells/ml} \tag{5-1}$$

Near the end of the exponential phase the bacteria achieved a concentration of approximately 6.5×10^{8} cells/ml. As shown in the following calculation, at this concentration, the bacteria were "stacked" an average of 21 cells deep.

$$\text{Area Occupied by all Bacteria} = \frac{6.5\times10^{8}\,\text{cells}}{\text{ml}}(4\text{ml})\frac{(0.62\ \mu\text{m})^{2}\pi}{\text{cell}} = 31\ \text{cm}^{2} \qquad (5\text{-}2)$$

$$\text{Layers of Bacteria} = \frac{\text{Area of Bacteria}}{\text{Surface Area of Septa}} = \frac{31\ \text{cm}^{2}}{1.5\ \text{cm}^{2}} = 21$$

This dense cell pack would certainly limit the amount of nutrients available to many bacteria, especially those at the bottom of the "stack". It is believed that this results in a lower final cell density at high acceleration levels. Also, because buoyancy forces increase with higher levels of acceleration, beneficial by-products are removed from the vicinity of the cells very quickly as acceleration increases. It is proposed that this also contributes to the inverse relationship between acceleration and final cell density.

The proposed model also accurately predicted the observed non-monotonic relationship between acceleration and the length of the lag phase. For simulated accelerations less than 1 *g*, it was hypothesized that the reduced effects of convection would allow higher concentrations of excreted by-products to remain around the bacteria. Considering the findings in Chapter 4, this was expected to result in a shorter lag phase than in 1 *g* controls.

The non-monotonic effects of hypergravity on the length of the lag phase were also predicted by the proposed model. At 50 *g*, sedimentation concentrated the bacteria near the bottom of the test tube within approximately 3.6 hours (reference Appendix A for calculations). It is proposed that this created a much higher concentration of excreted by-products around the bacteria, which resulted in a shorter lag phase than in either the 1 *g* controls or simulated hypogravity experiments.

However, it is believed that when the acceleration level was increased above approximately 100 *g*, buoyancy-driven convection caused the less dense by-products to rise to the surface at a faster rate. This resulted in a lower concentration of beneficial by-products around the bacteria, and the length of the lag phase started to increase. Eventually, around 400 *g*, the lag phase was equivalent to that of 1 *g* controls.

Although it was not hypothesized, the effects of hypogravity on the length of the exponential phase and the growth rate can possibly be explained by this model as well. Due to the reduced effects of sedimentation and buoyancy-driven convection on a clinostat or in space, it is conceivable that bacteria have a small volume of surrounding medium with a relatively low glucose concentration. If, due to the lack of convection, this is a relatively stable region, it might result in a slightly lower rate of glucose consumption. This is a similar condition to comparisons of fed-batch (low-glucose) cultures with single-batch (high-glucose) cultures. These comparisons have found fed-batch cultures have a longer exponential phase and higher final cell count than single-batch cultures (Robins and Taylor, 1989). It is therefore, proposed that a stable region around the cells cause the observed lower growth rate, and longer exponential phase for samples on a clinostat. It also may contribute to the higher final cell density relative to controls. In addition, because the cells on a clinostat remain colloidal, they have better nutrient availability than cells in 1 *g*. This could also help explain the observed longer exponential phase for cultures on a clinostat.

5.7 CONCLUSIONS

The experiments described in this chapter supported all four hypotheses, which were based upon the proposed new model concerning bacterial growth in different inertial environments. The clinostat experiments, which only simulated the indirect effects of reduced gravity, had similar results to previous space experiments. It is therefore reasonable to conclude that space flight has an indirect effect on suspended bacterial cultures, by altering the fluid environment surrounding the bacteria. It is believed that these changes are caused by two gravity-dependent forces -- sedimentation acting on the bacteria and buoyancy-driven convection acting on their excreted by-products. Simulations of 0 *g* with a clinostat, a novel method of simulating 0.5 *g* with an inclined clinostat, and centrifuge experiments of 50 *g*, 180 *g*, and 400 *g* all consistently supported the proposed model.

In summary, the following conclusions were made regarding the effects of acceleration on the three phases of bacterial growth.

5.7.1 Final Cell Density

Final cell density decreases as acceleration increases. It is postulated that this is caused by two gravity-dependent forces. As acceleration increases, sedimentation causes the cells to quickly accumulate at the bottom of the culture tube, which reduces their nutrient availability. Also, buoyancy-driven convection increases at higher levels of acceleration. This causes beneficial by-products to rapidly rise from the bacteria and further reduces the final cell density as acceleration increases.

5.7.2 Length of the Lag Phase

a. *Accelerations between simulated 0 g and 1 g:* The length of the lag phase is directly related to acceleration. This appears to be a linear relationship (or very close to linear). It is hypothesized that this is caused by the reduced effectiveness of convection during clinorotation. This allows beneficial by-products to remain near the cells, which shortens the lag phase.

b. *Accelerations between 1 g and 100 g:* Accelerations of approximately 50 *g* result in a significantly shorter lag phase than at any other acceleration level. Because the cells are clustered at the bottom of their culture tube, each bacterium is surrounded by very high concentrations of by-products, such as CO_2, which reduce the lag phase.

c. *Accelerations greater than 100 g:* As acceleration increases above approximately 100 *g*, the length of the lag phase increases. Eventually there is no difference relative to the length of the lag phase in 1 *g* controls. At these levels of acceleration, sedimentation clusters the bacteria at the bottom of the culture tube. Although this does tend to increase the concentration of by-products surrounding the cells, this is partially off-set by the effects of buoyancy-driven convection. As acceleration increases buoyancy-driven convection causes the by-products to move away from the cells at a faster rate, and this increases the length of the lag phase.

5.7.3 Growth Rate and Length of the Exponential Phase

On average, simulations of 0 *g* produced a 1.1 hour longer exponential phase and a slower growth rate than samples at 1 *g*. Simulations of 0.5 g had similar, yet less pronounced, differences relative to controls. It is believed that this was caused by the reduced effects of convection and sedimentation on a clinostat, which created a region of relatively low glucose concentration surrounding the bacteria.

CHAPTER 6

BUOYANCY FORCES ASSOCIATED WITH BACTERIAL GROWTH

6.1 ABSTRACT

The existence of buoyancy forces associated with bacterial growth was investigated through qualitative experiments and mathematical analysis. Qualitative data involved observing plumes rising in a liquid medium from many bacteria clustered at the bottom of a culture tube. Mathematical analysis included estimating the velocity, Reynolds number, and boundary layer associated with these plumes. All of these calculations agreed with observed results. Additional analysis estimated the plume velocity rising from a single cell is 30 times greater than the sedimentation rate of a single *E. coli* cell. Despite these high velocities, further analysis indicated that clinorotation is still a reasonable simulation of the absence of buoyancy forces on orbit. Analysis also showed this large force could explain the non-linear relationships observed between acceleration and growth kinetics. These results support the theory that bacterial growth causes convective currents in the medium immediately surrounding each cell, and these currents significantly affect the length of the lag phase and the final cell density in suspension cultures.

6.2 BACKGROUND

The proposed theory that density-driven convection, acting on bacterial by-products, affects bacterial growth is a new idea. However, this same buoyant force is the accepted physical mechanism that explains why larger crystals can be grown in space (Brailovskaya *et al.*, 1994; Fehribach and Rosenberger, 1989; McCay and McCay, 1994; Pusey and Naumann, 1986; Pusey *et al.*, 1986, 1988). As a crystal grows, the medium immediately surrounding it becomes less dense than the surrounding bulk fluid. In 1 *g* this results in convective flows around the crystal, which leads to inhomogeneous solute concentrations and poor growth conditions. However, in a microgravity environment there are no convective flows and the transport of solute is purely a diffusive process. This allows a more ideal growth environment for the crystal.

Plumes associated with crystal growth have been investigated in two general methods. A significant amount of research has focused on the mathematical equations associated with convective plumes rising from growing crystals (Brailovskaya *et al.*, 1994; Fehribach and Rosenberger, 1989; Ostrach, 1982, 1983; Pusey and Naumann, 1986; Pusey *et al.*, 1988, 1986; Yeoh *et al.*, 1987; Zhou and Zebib, 1994). Other research has measured the plume velocity experimentally using Schlieren photography (Naumann and Herring, 1980; Pusey *et al.*, 1988) or holograms (McCay and McCay, 1994). Of particular interest, the research by Pusey *et al.* (1988) compared their mathematical models with empirical measurements of rising plumes from crystals as

small as 10 μm in diameter. This study showed that the mathematical models were within an order of magnitude of observed results.

This chapter consists of a similar investigation of plumes associated with bacterial growth. First, a picture of a plume rising from a layer of growing bacteria is presented. The experimental data are followed by mathematical analysis of plumes associated with bacterial growth. Mathematical equations are used to first analyze convective plumes rising from bacteria clustered at the bottom of a culture tube. Then similar analysis is also applied to buoyancy forces associated with a single bacterium. The chapter concludes by explaining mathematically how these forces could explain the non-linear experimental results from Chapter 5.

6.3 QUALITATIVE EVIDENCE OF BUOYANCY FORCES

6.3.1 Specific Experimental Methods

General *E. coli* culture methods were described in Chapter 3. Specific to the experiments discussed in this chapter, two 5 ml samples of *E. coli* were cultivated in one FPA. The samples were separated by rubber septa. In an effort to reduce the time required to reach the stationary phase, the inoculation used was approximately 5×10^8 cells/ml. A few days later the samples had reached their stationary phase, and within a week the samples had sedimented to the bottom of their containers. At this time two additional 5 ml samples were inoculated in fresh medium in a second FPA. A day later these new samples were near the end of their exponential phase. Both FPAs were then

vortexed. This suspended the cells in the samples that had reached their stationary phase a week prior to the experiment. The FPAs were then both placed in a centrifuge at 700 *g* for one hour.

When the samples were removed from the centrifuge all the bacteria were at the bottom of their culture tube, and the media in both FPAs were clear. The FPAs were immediately placed on a table and photographed for approximately 5 minutes with back-lighting.

6.3.2 Results and Discussion

Thirty seconds after the samples were removed from the centrifuge a plume could clearly be seen in the FPA that was inoculated the previous day. However, no plume was visible in the two samples that had been inoculated a week before the experiment. This comparison is shown in Figure 6-1. This photograph, taken 30 seconds after the samples were removed from the centrifuge, shows a plume rising approximately 2.8 cm (average velocity of 0.09 cm/sec). The plume continued to be visible in both samples in the right FPA for about 10 minutes. After this time, the samples in the right FPA were too cloudy to continue observing any plume. In contrast, the FPA on the left, which contained roughly the same number of cells, never had a plume in either sample.

These findings support the proposed theory that bacterial growth creates buoyancy-driven convection. Diffusion and sedimentation alone can not explain these results. Because the samples in the right FPA were still growing, they were also consuming glucose. It is believed that this created a density gradient at the bottom of

these samples. As a result a plume rose from the less dense medium immediately surrounding the bacteria, and some cells were carried by the plume. As the bacteria were distributed throughout the samples in the right FPA, the media became cloudy. In contrast, the samples in the left FPA never had a plume, and never became cloudy, because these bacteria were no longer metabolizing glucose.

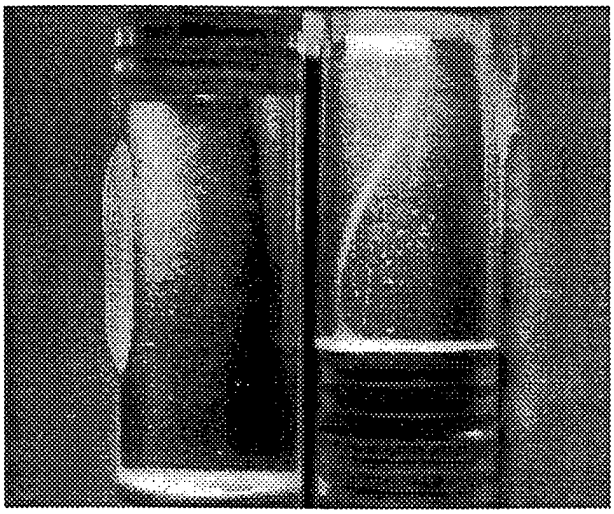

Figure 6-1 Plume Rising From Growing Bacteria. This photograph was taken 30 seconds after removing both samples from a centrifuge. The sample on the right, which shows a plume rising from the bottom, contained bacteria that were still growing. The sample on the left, which never had a plume, contained bacteria that had stopped metabolizing glucose.

6.4 MATHEMATICAL ANALYSIS OF A PLUME FROM MANY BACTERIA

6.4.1 Approach

Analysis of the plume shown in Figure 6-1 included estimating its velocity, Reynolds number, and boundary layer. All three of these parameters were expected to be within an order of magnitude of the observed results. The velocity was predicted to be approximately 0.09 cm/sec. The Reynolds number was hypothesized to be less than 2,000, indicating a laminar flow, and because the boundary layer was not visible, it was expected to be less than approximately 1 mm.

The plume velocity, Reynolds number, and boundary layer could not be calculated until some critical parameters were estimated. These included the solute and temperature gradients and the Lewis number, used for determining the relative roles of these gradients. The Grashof and Schmidt numbers then had to be calculated from these parameters. Only then could the plume velocity, Reynolds number, and boundary layer be estimated.

6.4.2 Estimating the Solutal Gradient

Fortunately, the values for most parameters required to find the solutal gradient were well known and did not have to be estimated. These and are listed below.

g = acceleration of gravity = 980 cm/sec

L = diameter of the plume's source (Pusey and Naumann, 1986; Pusey *et al.*, 1988)

For a plume from many bacteria at the bottom of an FPA,
L = the diameter of the FPA = 1.4 cm

ρ_∞ = density of bulk (fresh) medium = 1.011 g/cm^3 (Klaus *et al.*, 1997)

ρ_{H_2O} = density of water = 0.997 g/cm^3 (Reynolds and Perkins, 1977)

$$\nu = \text{medium's kinematic viscosity} = \frac{\text{viscosity}}{\text{density}} = \frac{3.6\ ^{kg}/_{hr \cdot min}}{1.011\ ^{g}/_{cm^3}} = 9.9 \times 10^{-3}\ \text{cm}^2/\text{sec}$$

(See Appendix A)

α = thermal diffusivity in water = 1.4×10^{-3} cm^2/sec (Reynolds and Perkins, 1977)

β = thermal volumetric expansion coefficient of water = 1.8×10^{-4} C^{-1} (Reynolds and Perkins, 1977)

Other parameters were not well known and had to be estimated. For example, the by-product diffusion coefficient was estimated based upon some common metabolites of *E. coli.* These are listed in Table 6-1 (Cussler, 1997; Atlas 1997). Based upon these values, *E. coli's* by-product diffusion coefficient was assumed to be 1.21×10^{-5} cm^2/sec, which is the diffusion coefficient for acetic acid. This is one of the most common extracellular by-products of *E. coli* (Roberts *et al.*, 1955), and its diffusion coefficient is close to the average value from Table 6-1.

Table 6-1 Diffusion Coefficients for By-Products of *E. coli*

By-Product	D in H_2O
Ethanol	8.4×10^{-6} cm^2/sec
Acetic acid	1.21×10^{-5} cm^2/sec
Formic acid	1.50×10^{-5} cm^2/sec
Carbon Dioxide	2.03×10^{-5} cm^2/sec

The density of the used medium also had to be estimated. This was accomplished knowing that approximately 30% (Roberts *et al.*, 1955) to 50% (Gottschalk, 1986) of the glucose consumed by *E. coli* is used to supply the carbon requirements for the cell. The rest of the glucose is converted to by-products that are excreted back into the surrounding medium. Assuming an average value of 40% of the glucose consumed is used for cell growth, the density of used medium was found to be,

$$\rho_{\text{Used}} = \rho_{\infty} - 0.4\left(\rho_{\infty} - \rho_{H_2O}\right) = 1.011 - 0.4\left(1.011 - 0.997\right) = 1.0054\ \text{g/cm}^3. \quad (6\text{-}1)$$

This assumes there is absolutely no glucose in the medium immediately surrounding a bacterium. For the case when all the bacteria are at the bottom of the FPA, this is a logical assumption. As shown in Equation 6-2, under these conditions cells are "stacked" an average of 40 bacteria high. It is therefore reasonable to assume that no glucose remains near the bacteria at the bottom of the "stack".

$$\text{Area Occupied by all Bacteria} = 1\times10^{9}\ \tfrac{\text{cells}}{\text{ml}}\left(5\text{ml}\right)\frac{\left(0.62\mu\text{m}\right)^2\pi}{\text{cell}} = 60\text{cm}^2$$
$$\text{Layers of Bacteria} = \frac{\text{Area of Bacteria}}{\text{Area of Bottom of the Culture Tube}} = \frac{60\text{cm}^2}{1.5\text{cm}^2} = 40 \quad (6\text{-}2)$$

Therefore, using the density of used medium from Equation 6-1,

$$\frac{\Delta\rho}{\rho_{\infty}} \approx \frac{1.011 - 1.0054}{1.011} = 0.0055. \quad (6\text{-}3)$$

6.4.3 Relative Effects from Thermal and Concentration Differences

Prior to calculating the plume velocity a determination had to be made concerning the relative effects of thermal expansion and solute concentration (Ostrach, 1982, 1983). In other words, what affects the density of the medium surrounding bacteria the greatest; is it the heat generated by growing bacteria, or the differences in solute concentration? An estimate of the density difference due to solute concentration was accomplished above, but *E. coli* also produces up to 3 kcal/l every hour (Bailey and Ollis, 1986). Both temperature and reduced solute concentrations reduce the surrounding medium's density. However, equations for estimating the plume velocity normally only consider the dominant effect, either thermal or concentration gradients.

The best way to answer this question was by comparing the ratio of concentration to thermal buoyancy, shown in Equation 6-4.

$$N \equiv \frac{\Delta\rho / \rho_o}{\beta \Delta T} \tag{6-4}$$

The numerator in Equation 6-4 is due to solute differences, and the denominator is due to thermal expansion (Ostrach, 1982, 1983; Pusey and Naumann, 1986). If $N >> 1$, density differences are primarily affected by solute gradients, and if $N << 1$, the density differences are primarily due to thermal effects. Unfortunately, it is difficult to estimate ΔT. However, we can calculate what ΔT must be for $N = 1$, which would mean the thermal and concentration differences have equivalent effects on the medium's density. Solving for ΔT in Equation 6-4, we find

$$\Delta T = \frac{\Delta\rho/\rho_o}{N\beta} = \frac{5.5\times10^{-3}}{1.8\times10^{-4}\,\mathrm{C}^{-1}} = 31^{\circ}\mathrm{C}. \qquad (6\text{-}5)$$

This means the medium surrounding bacteria at the bottom of an FPA would have to be 31°C greater than the temperature of the bulk fluid for buoyancy effects from thermal gradients to be equal to that caused by concentration differences. This unrealistically high temperature difference indicates that thermal effects are minimal. The density differences surrounding growing bacteria are most likely dominated by differences in solute concentration.

Additional insight can also be obtained by calculating the Lewis number. This is the ratio of the Schmidt and Prandtl numbers, which compares the thermal diffusivity to the by-product diffusion coefficient.

$$\text{Lewis Number} = \frac{\mathrm{Sc}}{\mathrm{Pr}} = \frac{\alpha}{D} = \frac{1.4\times10^{-3}\ \mathrm{cm}^2/\mathrm{sec}}{1.21\times10^{-5}\ \mathrm{cm}^2/\mathrm{sec}} = 116 \qquad (6\text{-}6)$$

This means the thermal boundary layer is much larger than the solutal layer, and the mass diffusion process is much slower than thermal convection (Ostrach, 1983). This is because the heat produced by growing bacteria diffuses much faster than the excreted by-products. As a result the sample is essentially isothermal.

6.4.4 Plume Velocity, Reynolds Number, and Boundary Layer from Many Bacteria

We can now calculate the plume velocity from many bacteria, assuming density gradients are due primarily to differences in solute concentration. This is

accomplished using Equation 6-7 (Pusey and Naumann, 1986; Pusey *et al.*, 1988, 1986).

$$V = \left(\frac{Gr}{Sc}\right)^{1/2} \frac{\nu}{L} \tag{6-7}$$

where,

Gr = the Grashof number (ratio between buoyancy and frictional forces)

$$= \frac{gL^3 \frac{\Delta\rho}{\rho}}{\nu^2} = \frac{980 \frac{\text{cm}}{\text{sec}^2}\left(1.4 \text{ cm}\right)^3 5.5\times10^{-3}}{\left(9.9\times10^{-3} \frac{\text{cm}^2}{\text{sec}}\right)^2} = 1.5\times10^5 \tag{6-8}$$

Sc = the Schmidt number (ratio of dynamic and diffusion boundary layers)

$$= \frac{\nu}{D} = \frac{9.9\times10^{-3} \frac{\text{cm}^2}{\text{sec}}}{1.21\times10^{-5} \frac{\text{cm}^2}{\text{sec}}} = 820$$

Substituting the values from Equations 6-8 into Equation 6-7, the apex plume velocity caused by a concentration of many bacteria can be calculated.

$$V = \left(\frac{1.5\times10^5}{820}\right)^{1/2} \frac{9.9\times10^{-3} \frac{\text{cm}^2}{\text{sec}}}{1.4 \text{ cm}} = 0.096 \frac{\text{cm}}{\text{sec}} \tag{6-9}$$

As expected, this velocity, based upon mathematical modeling, is very close to the observed velocity from Figure 6-1 of 0.09 cm/sec .

The Reynolds number associated with this rising plume indicates that the plume should be in laminar flow (Reynolds and Perkins, 1977).

$$\text{Re} = \frac{\rho \ell V}{\mu} = \frac{1.011 \frac{\text{g}}{\text{cm}^3}\left(2.8 \text{ cm}\right)0.096 \frac{\text{cm}}{\text{sec}}}{0.01 \frac{\text{g}}{\text{cm}\cdot\text{sec}}} = 27 \tag{6-10}$$

This is also consistent with the observed results shown in Figure 6-1.

Finally, the boundary layer was estimated to be approximately 0.1 mm from Equation 6-11 (Pusey and Naumann, 1986). While it is impossible to measure the observed boundary layer from Figure 6-1, this is a reasonable value considering a boundary layer was not visible during the experiment.

$$\delta = \frac{L}{(GrSc)^{1/4}} = \frac{1.4 \text{ cm}}{(1.5 \times 10^5 \cdot 820)^{1/4}} = 130 \mu\text{m} = 0.13 \text{ mm} \qquad (6\text{-}11)$$

These mathematical results were based upon the reasonable assumed values for D and $\Delta\rho$, which were discussed previously. Using these estimates, the plume velocity caused by many bacteria growing at the bottom of a culture tube was estimated, as well as the Reynolds number and boundary layer associated with the plume. All three of these values agreed with observed results.

6.5 MATHEMATICAL ANALYSIS OF A PLUME FROM ONE BACTERIUM

Using these same equations, the plume velocity from a single bacterium was also estimated. Unfortunately, unlike the calculations above, there were no observed results to compare with the analysis of the plume from one bacterium. However, two general hypotheses were made. First, based upon the computer simulation results discussed in Chapter 4, it was hypothesized that the plume velocity from one bacterium would be significantly greater than *E coli's* sedimentation rate of 0.06 μm/sec (see Appendix A). In addition, because the results in Chapter 5 showed that clinorotation produced similar results to those in space flight, the plume velocity was

also expected to be slow enough to allow the by-products to remain around the bacteria during clinorotation.

In calculating the plume velocity from a single bacterium, it was assumed that Equation 6-7 is valid for plumes as small as 1 μm in diameter. Although there is no reason to believe the equation can not be applied to a single bacterium, it has only been validated for plumes as small as 10 μm in diameter (Pusey *et al.*, 1988).

6.5.1 Values of Parameters

Only two parameters from the previous calculations had to be changed when addressing the plume from a single bacterium. The plume's diameter, L, was no longer the diameter of an FPA, but was instead the diameter of a bacterium (1.2 μm). Also, the density difference estimated in Equations 6-1 and 6-3 was not valid for one bacterium. This estimate assumed no glucose remained in the medium immediately surrounding the bacteria. This was reasonable for bacteria clustered at the bottom of a culture tube, but it is not valid for a single bacterium.

This is evident by calculating the Damköhler number for *E. coli*, which is a ratio of the reaction rate to the mass transfer rate for glucose to the cell (Bailey and Ollis, 1986).

$$\mathrm{Da} = \frac{\text{maximum reaction rate}}{\text{maximum mass transfer rate}} = \frac{v_{max}}{k_s s_0} \qquad (6\text{-}12)$$

where

v_{max} is the maximum reaction rate, estimated using the following parameters:
- *E. coli* doubles in 2.5 hours at 23° C ; so its mass increases 10^{-12} grams in 9,000 seconds.
- Approximately 40% of the glucose consumed is used for cellular growth.

- *E. coli's* surface area is 5.0×10^{-8} cm^2 (See Appendix A).

$$v_{max} \approx \frac{1}{0.40}\left(\frac{10^{-12}\ \frac{g}{\text{bacterium}}}{9{,}000\ \text{sec}}\right)\frac{\text{bacterium}}{5.0\times10^{-8}\,\text{cm}^2} = 5.6\times10^{-9}\ \frac{g}{\text{cm}^2\text{sec}} \qquad (6\text{-}13)$$

k_s is the mass transfer coefficient, which was estimated assuming glucose diffuses to the cell over a distance of 10 μm (0.001 cm) (Bailey and Ollis, 1986).

$$k_s = \frac{D_{\text{Glucose}}}{\text{Diffusion distance}} \approx \frac{6.0\times10^{-6}\ \frac{\text{cm}^2}{\text{sec}}}{0.001\ \text{cm}} = 0.006\ \frac{\text{cm}}{\text{sec}} \qquad (6\text{-}14)$$

S_0 is the concentration of glucose in the bulk fluid ≈ 5.0 g/l = 0.005 g/cm^3.

Substituting all of these values into Equation 6-12, the Damköhler number for a single bacterium of *E. coli* was found to be much less than one.

$$\text{Da} = \frac{v_{max}}{k_s s_0} \approx \frac{5.6\times10^{-9}\ \frac{g}{\text{cm}^2\text{sec}}}{0.006\ \frac{\text{cm}}{\text{sec}}\left(0.005\ \frac{g}{\text{cm}^3}\right)} = 1.9\times10^{-4} \qquad (6\text{-}15)$$

This shows that the consumption of glucose by a single bacterium is reaction limited, not diffusion limited. Therefore, it is unreasonable to assume that 100% of the medium immediately surrounding a single bacterium is composed of by-products, without any glucose.

A more reasonable assumption is that the concentration of by-products surrounding each bacterium, X_B, is roughly 5%. This means the remaining 95% of the surrounding medium is assumed to contain glucose. Based upon this estimate, and the result of Equation 6-3,

$$\frac{\Delta\rho}{\rho_\infty} = X_B\left(\frac{\Delta\rho}{\rho_\infty}\ \text{if all glucose is consumed}\right) \approx 0.05\left(0.0055\right) = 2.8\times10^{-4}. \qquad (6\text{-}16)$$

This approximation was used for the initial calculations. Additional calculations were later performed using values for X_B from 0% to 100%.

6.5.2 Plume Velocity from One Bacterium

Using these values, the Grashof number for the plume from one bacterium was found to be

$$Gr = \frac{gL^3 \frac{\Delta\rho}{\rho}}{\nu^2} = \frac{980 \frac{cm}{sec^2}\left(1.2 \times 10^{-4} cm\right)^3 2.8 \times 10^{-4}}{\left(9.9 \times 10^{-3} \frac{cm^2}{sec}\right)^2} = 4.8 \times 10^{-9}. \qquad (6\text{-}17)$$

The initial plume velocity was then estimated.

$$V = \left(\frac{Gr}{Sc}\right)^{1/2} \frac{\nu}{L} = \left(\frac{4.8 \times 10^{-9}}{820}\right)^{1/2} \frac{9.9 \times 10^{-3} \frac{cm^2}{sec}}{1.2 \times 10^{-4} cm} = 2.0 \times 10^{-4} \frac{cm}{sec} = 2.0 \frac{\mu m}{sec} \qquad (6\text{-}18)$$

Figure 6-2 shows this result, as well as similar calculations using different values for X_B and different by-product diffusion coefficients. As expected, almost all of the velocities in Figure 6-2 are significantly greater than *E. coli's* sedimentation rate of 0.06 μm/sec. In fact, the plume velocity is less than 0.06 μm/sec only for values of X_B less than 0.005%. This indicates that buoyancy-driven convection is the dominant force involved in separating bacteria from their by-products, not sedimentation.

It is important to realize, however, that this is only the initial velocity. Unlike the plume rising from many bacteria clustered at the bottom of a culture tube, the plume from one bacterium expands as it rises. As the plume expands its velocity also decreases. This is depicted graphically in Figure 6-3.

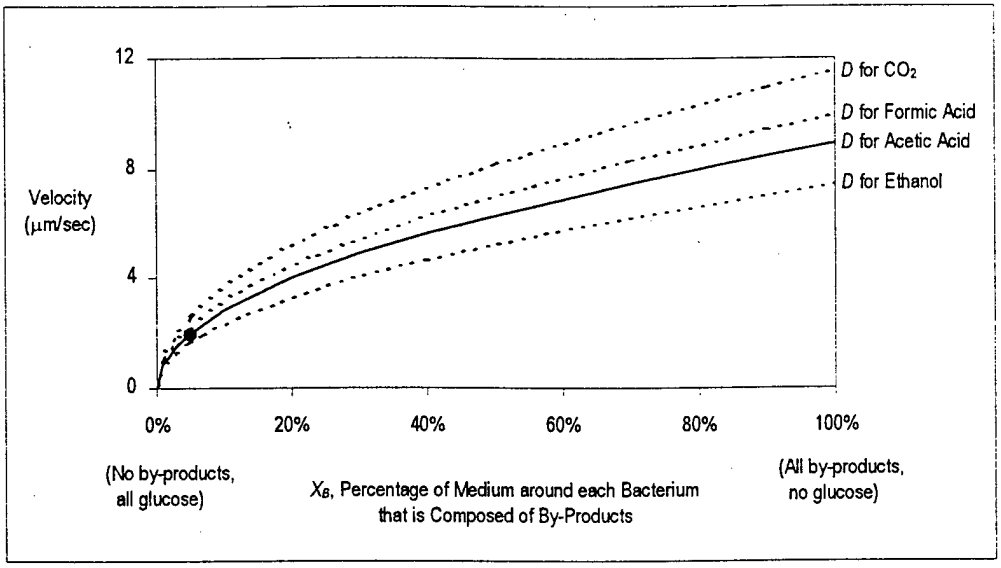

Figure 6-2 Initial Plume Velocity from One Bacterium. Velocities are shown for different values for X_B and different diffusion coefficients. The ● identifies the velocity calculated in Equation 6-18. Almost all velocities in the figure are much greater than *E. coli's* sedimentation rate of 0.06 μm/sec.

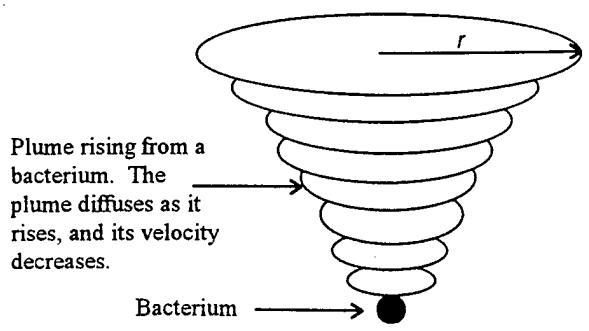

Figure 6-3 Expanding Plume as it Rises from One Bacterium in 1 *g*

There are a few reasons an expanding plume is significant. First, it is believed that small convective currents carry by-products away from each cell. However, by the time a plume has risen far enough to reach the next bacterium (a distance of approximately 120 μm) it should have diffused considerably. Therefore, the high concentration of by-products carried away from one bacterium are not expected to be offset by additional by-products rising from other bacteria.

The expanding plume is also significant because the plume's velocity decreases as it rises. Cussler (1997) showed that the velocity of an expanding plume at any height, z, is proportional to $z^{-1/3}$. Using this relationship, numerical integration allowed the plume's velocity to be determined as a function of time. This was accomplished by assuming the initial velocity of 2.0 μm/sec, found in Equation 6-18, occurs at a height of 0.62 μm above the bacterium's center (one radius above the bacterium). This means the plume's velocity is a maximum when it is even with the highest portion of the bacterium. The velocity then decreases as the plume rises.

As shown in Figure 6-4, the estimated plume velocity decreases rapidly. However, even 6 hours after a plume leaves a bacterium (not shown in Figure 6-4), its velocity is still considerably greater than the bacterium's sedimentation rate.

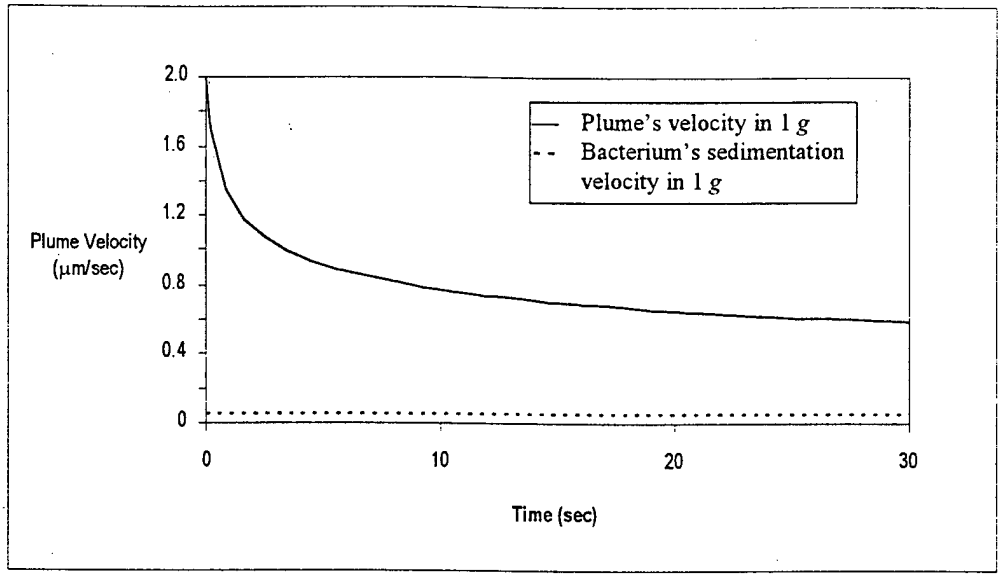

Figure 6-4 Estimated Plume Velocity from One Bacterium as a Function of Time. The plume's velocity decreases rapidly as it rises from a single bacterium. However, it is still much greater than a bacterium's sedimentation rate.

6.5.3 Effectiveness of Clinorotation for Simulating Hypogravity

As expected, the estimated plume velocity from one bacterium is considerably greater than the cell's sedimentation rate of 0.06 μm/sec. However, this raises an important question. Is the plume velocity so high that clinorotation is ineffective? The experiments discussed in Chapter 5 used a clinostat rotating at approximately 8 RPM. This caused the "downward" direction to constantly change, and the cells actually sedimented in a small circle (reference Figure 3-1). Because this circle had a radius less than the bacteria's diffusion distance, the cells were in a state of "functional weightlessness". However, it is now important to determine if the plume also remains near the cell during clinorotation, as it would in microgravity. Because the clinostat samples consistently showed similar growth kinetics to previous space-flight

experiments, it was hypothesized that the plume's velocity is slow enough to allow the by-products to remain near each cell during clinorotation.

Using the relationship between velocity and time, shown in Figure 6-4, the position of a plume was calculated in two dimensions as a function of time. Because the magnitude of the plume's velocity decreases with time, it spirals away from the bacterium instead of moving in a perfect circle (see Figure 6-5). After approximately 3.4 seconds, the plume is almost 3 μm from its original location. This is equivalent to the bacterium's expected displacement due to diffusion after one rotation, lasting 7.5 sec (reference Equation 3-4).

Figure 6-6 shows how the plume's distance from the original location varies with time. This is compared with the cell's expected diffusion distance and also the plume's distance from the origin without clinorotation. With the exception of the first 4.5 seconds, clinorotation keeps the plume within the cell's 1σ diffusion distance. In contrast, without clinorotation the plume moves away from its original location much faster than the bacterium diffuses. This indicates that the plume's velocity, although significantly greater than a bacterium's sedimentation rate, is still slow enough to allow less dense by-products to remain relatively near a bacterium during clinorotation. Therefore, clinorotation appears to be a reasonable simulation of the absence of buoyancy forces on orbit. However, it is not an ideal simulation. The by-products do move farther from a bacterium under clinorotation than they would on orbit. This

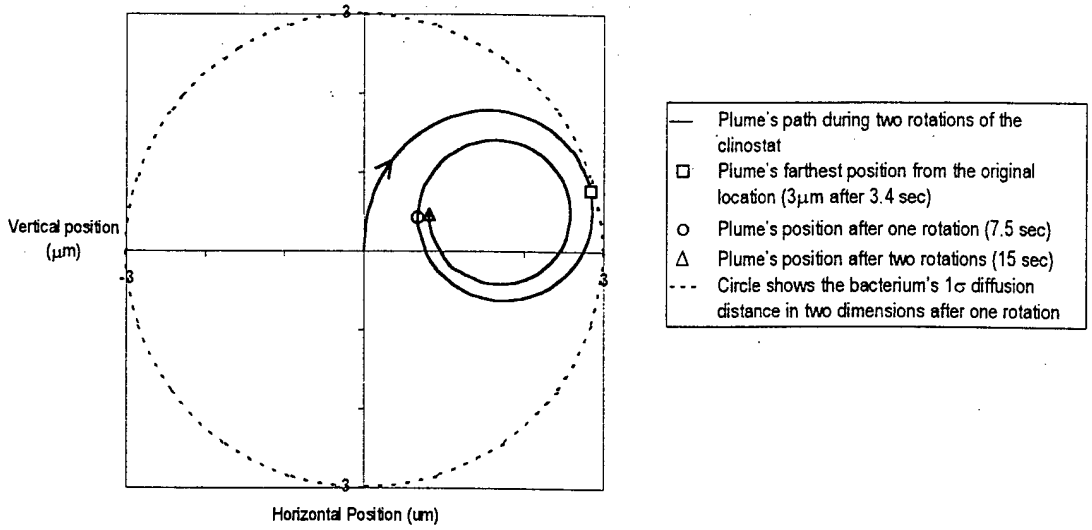

Figure 6-5 Position of a Plume Rising from a Bacterium during Clinorotation. Similar to Figure 3-1, this figure shows a non-rotating coordinate frame looking at the end of a culture tube. The origin is the bacterium's original location. As the clinostat rotates counter-clockwise, the plume spirals away from its original location.

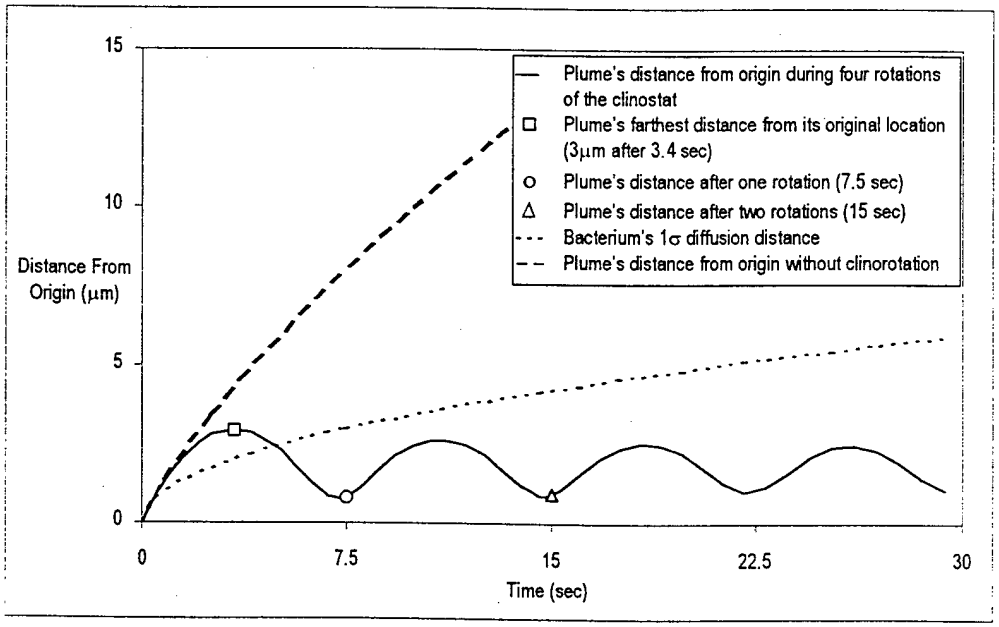

Figure 6-6 Plume's Distance from Origin Compared with the Cell's Diffusion Distance. In general, clinorotation keeps the plume within the cell's diffusion distance. Without clinorotation, however, the plume carries by-products much farther from the cell. Data points and curves shown are consistent with data in Figures 6-4 and 6-5.

could help explain why the clinostat experiments in this dissertation did not have as dramatic of differences as previous space-flight experiments when compared with 1 *g* controls.

6.6 EFFECTS OF HYPERGRAVITY ON BUOYANCY FORCES

The previous analysis indicated that a clinostat can reasonably simulate hypogravity, even when considering the relatively high plume velocity. This section addresses the effects of simulated hypergravity on the plume's velocity and how this can help explain the non-linear relationships between acceleration and growth kinetics documented in Chapter 5.

It was proposed in Chapter 5 that at accelerations above approximately 10 *g*, sedimentation quickly concentrates bacteria at the bottom of their culture tube. This is believed to initially be beneficial for growth because the cells are surrounded by the by-products from many bacteria. However, as the acceleration level increases so does buoyancy-driven convection. This causes the less dense conditioned medium to move away from the bacteria at a faster rate. This was expected to reduce the lag phase and also contribute to a lower final cell density as acceleration increases above 100 *g*.

The experimental results from Chapter 5 supported these hypotheses. The effects of acceleration between 0 *g* and 110,000 *g* were summarized in Figures 5-4 and 5-5. The same data are displayed in Figures 6-7 and 6-8 using a slightly different format. Both of these figures show only the effects from accelerations between 1 *g* and 1,000 *g*, and they do not use a semi-log scale. Also, the ordinate axis in Figure 6-

7 is reversed, so the axis moves from the highest final cell density (at 0 g) to the lowest final cell density (at 1,000 g).

It is proposed that this data can be explained by the relationship between acceleration and plume velocity. The equation for plume velocity, given in Equation 6-7, can be reduced to

$$V = \left(\frac{Gr}{Sc}\right)^{1/2} \frac{\nu}{L} = \left(\frac{gL\frac{\Delta\rho}{\rho_\infty}D}{\nu}\right)^{1/2}. \qquad (6\text{-}19)$$

This shows that plume velocity is a function of $g^{1/2}$, which is depicted graphically in Figure 6-9. This figure shows the plume velocity from many bacteria at the bottom of a culture tube as a function of acceleration. Notice, Figure 6-9 is very similar to Figures 6-7 and 6-8, particularly for accelerations above 10 g.

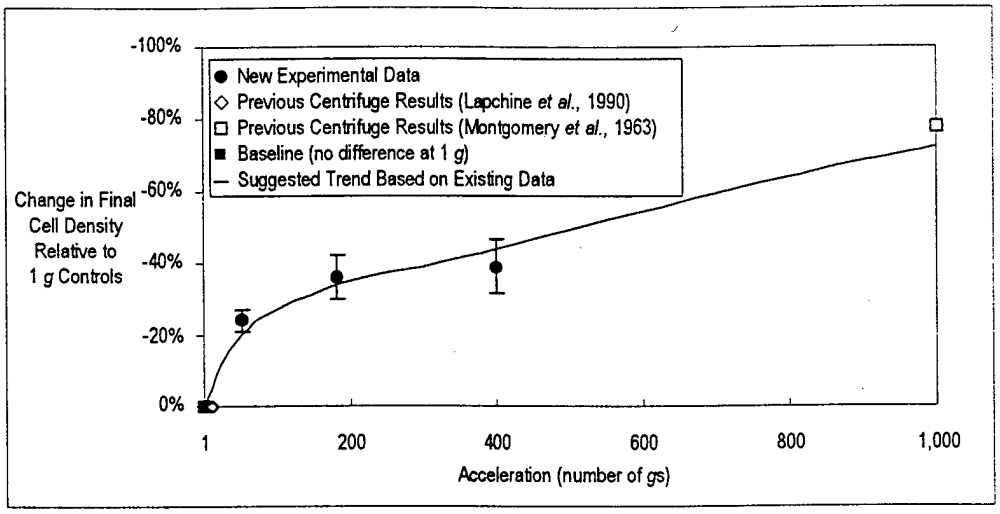

Figure 6-7 Effect of Accelerations between 1 *g* and 1,000 *g* on the Final Cell Density. As acceleration increases, final cell density decreases, but it does so at a decreasing rate.

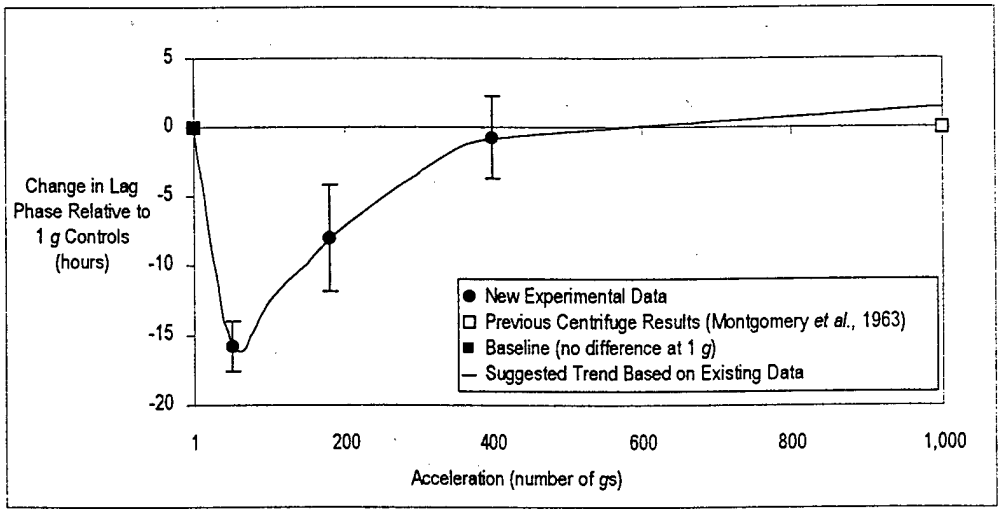

Figure 6-8 Effect of Accelerations between 1 *g* and 1,000 *g* on the Length of the Lag Phase. As acceleration increases above 50 *g*, the length of the lag phase increases, but it does so at a decreasing rate.

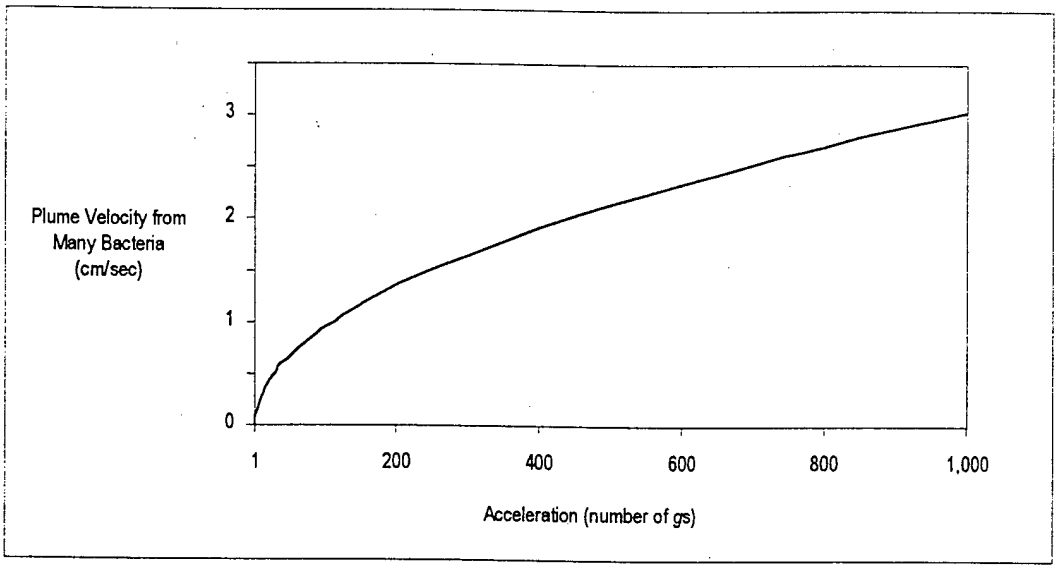

Figure 6-9 Effect of Acceleration on Plume Velocity from Many Bacteria. As acceleration increases the plume velocity also increases, but it does so at a decreasing rate. This curve is very similar to the relationship between acceleration and growth kinetics shown in Figures 6-7 and 6-8.

It is proposed that acceleration affects plume velocity, which changes the concentration of conditioned medium surrounding the bacteria. This directly affects the final cell density and the length of the lag phase. At an acceleration of 10 *g* all the bacteria sediment to the bottom of the culture tube within approximately 8 hours (see Appendix A). It is believed that this creates a concentration of preconditioned medium around the bacteria, which stimulates growth resulting in a shorter lag phase. However, after the bacteria begin to multiply, they become stacked on top of each other. This crowding is believed to restrict growth, resulting in a lower final cell density than 1 *g* controls.

At higher accelerations two things happen -- the bacteria sediment to the bottom of the culture tube sooner, and the excreted by-products rise to the top of the culture tube at a faster rate. Accelerations of 50 *g* and 400 *g* concentrate the bacteria at the bottom of the test tube within 3.6 hours and 0.5 hours, respectively. Although this is faster than the 8 hours required for complete sedimentation at 10 *g*, it is a relatively insignificant change considering that the length of the lag phase is approximately 25 hours. A more significant effect of higher accelerations is the increase in plume velocity, shown in Figure 6-9. It is believed that the higher plume velocity results in lower concentrations of conditioned medium around the bacteria. It is proposed that this restricts bacterial growth, resulting in a longer lag phase and also a lower final cell density as the acceleration increases. It is, therefore, proposed that there is a linear relationship between plume velocity and the change in final cell density and the change in lag phase for accelerations above approximately 10 to 50 *g*. More specifically, final cell density is hypothesized to be inversely proportional to plume velocity, and the length of the lag phase should be directly proportional to plume velocity. Considering this proposed relationship, and knowing plume velocity is a function of $g^{1/2}$, final cell density should be a function of $g^{-1/2}$, and the length of the lag phase should be a function of $g^{1/2}$, for accelerations greater than 10 to 50 *g*. This could explain the observed non-linear relationships between accelerations above 10 *g* and growth kinetics, shown in Figures 6-7 and 6-8.

To test this theory, the change in final cell density and change in lag phase, shown in Figures 6-7 and 6-8, were plotted as a function of plume velocity. If, as

hypothesized, plume velocity causes the non-linear effects of hypergravity on growth kinetics, there should be a linear relationship between plume velocity and the changes in final cell density and the length of the lag phase.

Figure 6-10 shows the change in final cell density as a function of plume velocity. This plot shows the same data points and trend line from Figure 6-7, only they are now both plotted as a function of plume velocity, not acceleration. The trend line, is very close to linear, suggesting that final cell density is inversely proportional to plume velocity for accelerations greater than about 10 *g*. This supports the proposed theory that above 10 *g* there is a linear relationship between final cell density and plume velocity, and final cell density is a function of $g^{-1/2}$.

Figure 6-11 shows the change in lag phase as a function of plume velocity. This figure uses the exact same data and trend line shown previously concerning the effect of acceleration on the lag phase. The only difference is this now the change in lag phase is shown as a function of plume velocity, not acceleration. This figure shows that accelerations of 50 *g* (and plume velocity of approximately 0.7 cm/sec) results in a much shorter lag phase than in 1 *g* controls. As previously discussed, it is believed that this is caused by sedimentation, which clusters the cells together. As acceleration increases, so does the plume velocity and the length of the lag phase. For accelerations between 50 *g* and 400 *g* there is almost a perfect linear relationship between plume velocity and the length of the lag phase. This clearly supports the proposed theory, indicating that the length of the lag phase is directly proportional to

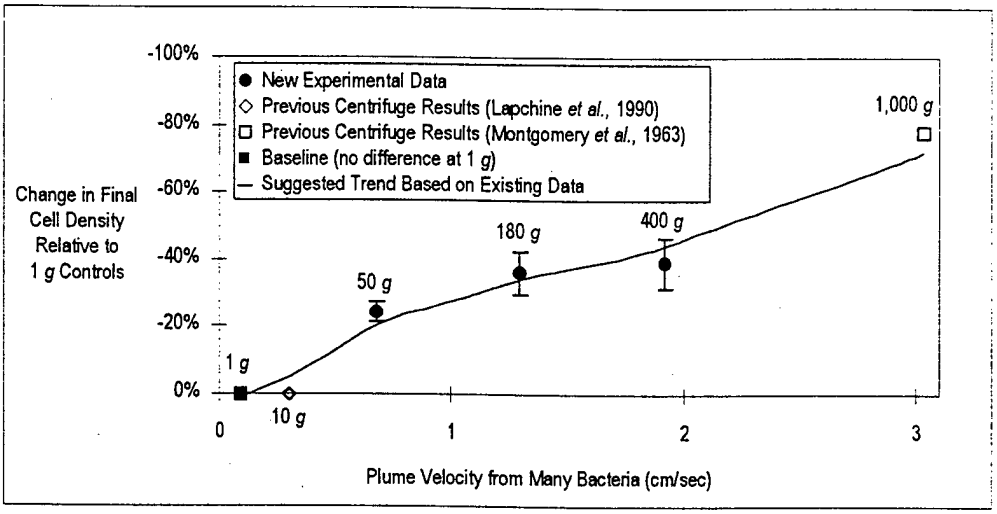

Figure 6-10 Change in Final Cell Density as a Function of Plume Velocity. The change in final cell density is inversely proportional to plume velocity for accelerations greater than about 10 *g*.

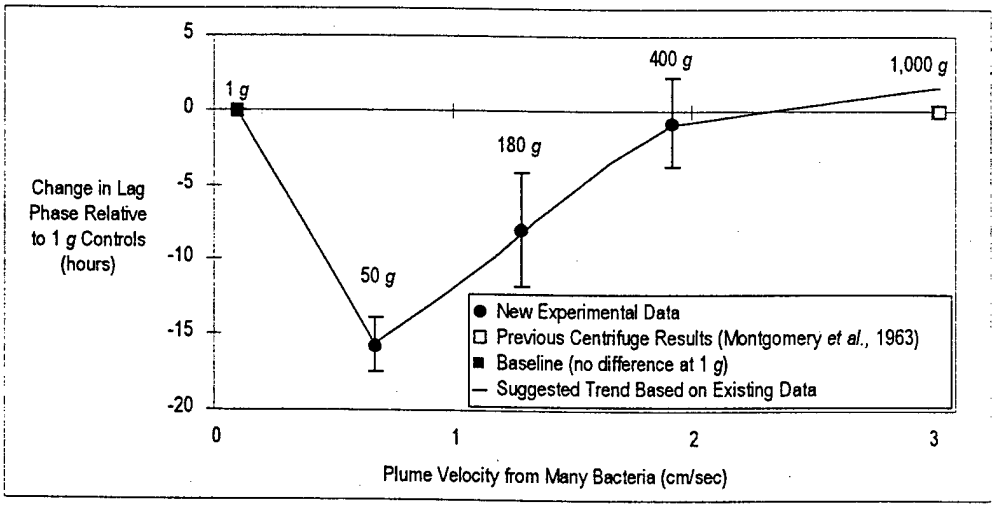

Figure 6-11 Change in the Length of the Lag Phase as a Function of Plume Velocity. The length of the lag phase is directly proportional to plume velocity for accelerations between 50 *g* and 400 *g*. However, the data point at 1,000 *g*, does not follow this linear trend. This is possibly due to the different materials and methods used by Montgomery *et al.* (1963).

plume velocity and both are a function of $g^{1/2}$. However, the single data point for a plume velocity of 3 cm/sec (corresponding to 1,000 g) does not follow this same linear relationship. This is possibly because this experiment, conducted by Montgomery *et al.* (1963), involved different materials and methods. This included cultivating *E. coli* at an average temperature that was 9°C warmer than the experiments in this dissertation.

6.7 CONCLUSIONS

All of the experimental and analytical analysis supported the proposed theory that buoyancy-driven convection affects bacterial growth. As hypothesized, visual observations indicated that growing bacteria create density gradients in their surrounding medium. Mathematical analysis of the plume velocity, Reynolds number, and boundary layer resulting from bacteria clustered at the bottom of an FPA were all very close to observed values. Additional analysis estimated the initial plume velocity from a single bacterium is 2 μm/sec. As expected, this is significantly greater than the *E. coli* sedimentation rate of 0.06 μm/sec. However, the plume velocity is still slow enough for clinorotation to reasonably simulate "functional weightlessness".

Additional analysis showed the relationship between plume velocity is a function of $g^{1/2}$. The data indicates that for accelerations above 10 g, final cell density is inversely proportional to plume velocity and is therefore a function of $g^{-1/2}$. The length of the lag phase appears to be directly proportional to plume velocity and a function of $g^{1/2}$ for accelerations above 50 g.

CHAPTER 7

EFFECTS OF SPACE FLIGHT, CLINOROTATION, AND CENTRIFUGATION ON THE METABOLISM OF *E. coli*

7.1 ABSTRACT

Two ground experiments and one space-flight experiment were conducted to determine the effects of inertial acceleration on the metabolism of *E. coli*. The ground experiments used a clinostat to simulate "functional weightlessness" and a centrifuge to achieve 50 *g*. At various times during growth, cell counts and glucose analyses were performed, and treated cultures were compared with 1 *g* controls. The biomass yield (mass of new cells normalized per mass of glucose consumed) from samples on the clinostat averaged 7.3% higher than controls ($p = 0.035$). The biomass yield from the samples on the centrifuge averaged 35.5% lower than controls ($p < 0.01$). A similar experiment on orbit found that space flight resulted in a 25.4% higher biomass yield than comparable ground controls ($p < 0.01$). All of these results were statistically significant and support the proposed theory that bacterial growth creates buoyancy-driven convection in the depletion zone surrounding each cell. Because natural convection does not occur on orbit, by-products that are beneficial for growth remain near the cells. This is believed to result in a more efficient metabolism of

nutrients for accelerations less than 1 *g*. In contrast, at hypergravity the buoyancy forces are believed to quickly separate these by-products from the bacteria, resulting in a lower biomass yield than in 1 *g* controls.

7.2 BACKGROUND

Many experiments have indicated that space flight stimulates the growth of bacterial cultures (Klaus *et al.*, 1997; Manko *et al.*, 1987; Mennigmann & Lange, 1986; Thévenet *et al.*, 1996; Ciferri *et al.*, 1986; Kordium *et al.*, 1980; Mattoni, 1968). A recent space-flight experiment also found that the bacterium *Streptomyces plicatus* produced 115% more of the antibiotic actinomycin D when compared with similar ground controls (Lam *et al.*, 1999). It is not yet know, however, if these increases in growth and yield are accompanied by a proportionally higher nutrient consumption, or if space flight allows a more efficient consumption of nutrients. This chapter addresses this question, and attempts to determine how inertial acceleration affects *E. coli's* biomass yield (mass of new cells per mass of nutrient consumed).

Three studies have previously addressed nutrient consumption on orbit. One of these experiments involved bacterial growth; the other two studied the nutrient consumption by a yeast and by human lung cells on orbit.

The only known experiment that investigated the effects of acceleration on bacterial nutrient consumption was conducted by Bouloc and D'Ari (1991). They reported that there was no difference in the cell growth or nutrient consumption for 3 *g* or 5 *g* experiments when compared with 1 *g* controls. They also compared the

growth of *E. coli* on orbit with similar ground controls using two different carbon-limiting media -- glucose and glycerol. For both media, they found there was no difference in the amount of nutrients consumed on orbit. However, contrary to almost all other reports, they observed no difference in the amount of bacterial growth in space. Therefore, their experimental results are unable to determine if increased growth on orbit is accompanied by a corresponding increase in nutrient consumption.

Walther *et al.* (1996) studied the cultivation of the yeast *Saccharomyces cerevisiae* in space. They found there was no difference in the cell cycle, cell proliferation, or glucose consumption on orbit when compared with similar ground controls. Contrary to this report, Montgomery *et al.* (1978) found that less glucose was consumed by human embryonic lung cells on orbit when compared with similar ground controls. On average the flight samples consumed 37% less glucose than the ground controls. They found no other differences in the two samples.

7.3 EXPERIMENT AND HYPOTHESES

Ground and flight experiments were conducted to determine the effects of inertial acceleration on bacterial metabolism. Based upon the proposed model of buoyancy-driven convection associated with bacterial growth it was hypothesized that bacteria metabolize nutrients more efficiently at lower levels of acceleration. As explained in the previous chapters, it is proposed that as inertial acceleration increases by-products are carried away from the cells by buoyancy-driven convection. Some of these by-products are believed to be beneficial for growth. These could include

cofactors, enzymes, or required products of anaplerotic sequences (Gottschalk, 1986). Without these by-products near the cells, the metabolism of nutrients becomes less efficient at hypergravity than at 1 *g*. However, in real or simulated microgravity the by-products remain around the bacteria, allowing a more efficient metabolism of nutrients. This is believed to increase the biomass yield for experiments at reduced levels of acceleration. This hypothesis is stated below and illustrated in Figure 7-1.

The mass of new cells normalized per mass of glucose consumed is inversely related to acceleration (including real and simulated hypogravity).

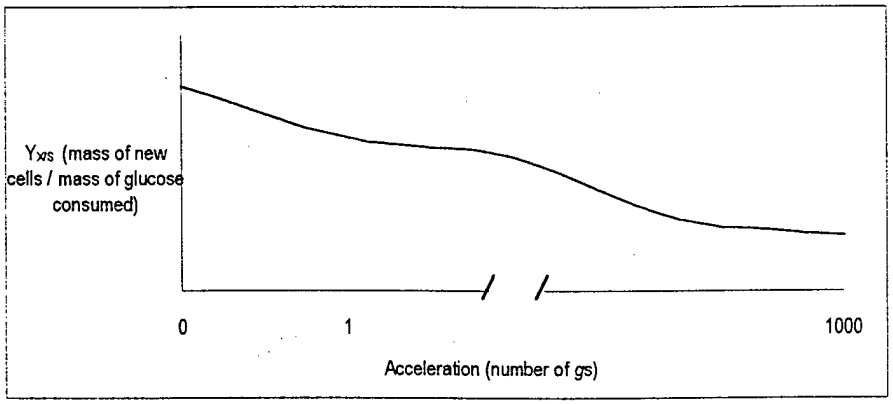

Figure 7-1 Hypothesized Effect of Acceleration on the Specific Biomass Yield

7.4 SPECIFIC MATERIALS AND METHODS

7.4.1 Ground Experiments

Two series of ground experiments were conducted using a clinostat to simulate "functional weightlessness" (Klaus *et al.*, 1998b) and a centrifuge to achieve 50 *g*.

Controls were kept at 1 *g*. As explained in Chapter 3, sample cultures were grown anaerobically in Vogel-Bonner "Medium E" minimal growth medium (Vogel and Bonner, 1956) containing approximately 5.9 ± 0.25 g/l of glucose. The inoculum of 10^6 cells/ml was taken from a saturated culture, grown in Medium E without glucose.

Approximately every 10 hours, 2 to 6 samples were collected from the different inertial conditions. Cell densities and logistic growth curves were determined using the general methods outlined in Chapter 3. The mass of new cells was then determined by multiplying the number of cells by the average mass of an *E. coli* bacterium (1×10^{-12} g/cell) (Klaus, *et al.*, 1997).

After removing the bacteria with a 0.22 μm filter, the samples were frozen and shipped overnight with solid CO_2 to Bristol-Myers Squibb Research Institute, for blind glucose analysis. This was accomplished with chromatography using a Hewlett-Packard model 1090 HPLC with an evaporative light scattering detector (gain = 8). A YMC-Pack, Polyamine II, S 5-μm-particle column (250 × 4.6 mm) was used with a solvent system of water and acetonitrile (1:3 by volume) and a flow rate of 1 ml/min. The volume of each injection was 0.02 ml. The concentration of glucose in each sample was subtracted from the average glucose concentration from fresh samples prior to inoculation. This difference gave the mass of glucose consumed by each sample. The biomass yield was then determined by dividing the mass of new cells produced by the mass of glucose consumed for every sample.

7.4.2 Flight Experiment

Four samples of *E. coli* were grown on STS-95 in October 1998. As with previous ground experiments, the bacteria were grown anaerobically in sterile FPAs with movable septa to confine the fluids. Additional protocol was also required for the flight experiment. Unlike the ground experiments, the inoculum was initially separated from the growth medium (reference Figure 7-2). Chamber A contained 3.5 ml of sterile Medium E with 7.8 $\pm$ 0.8 g/l of glucose. Chamber B held the inoculum of 8×10^6 cells/ml in Medium E without glucose. Approximately 2.5 hours after launch, the septa were pushed forward by a plunger allowing the inoculum to mix with the growth medium via the FPA by-pass. Chamber A then contained approximately the same concentration of bacteria and nutrients used for previous ground studies -- 10^6 cells/ml in Medium E with 6.8 g/l of glucose.

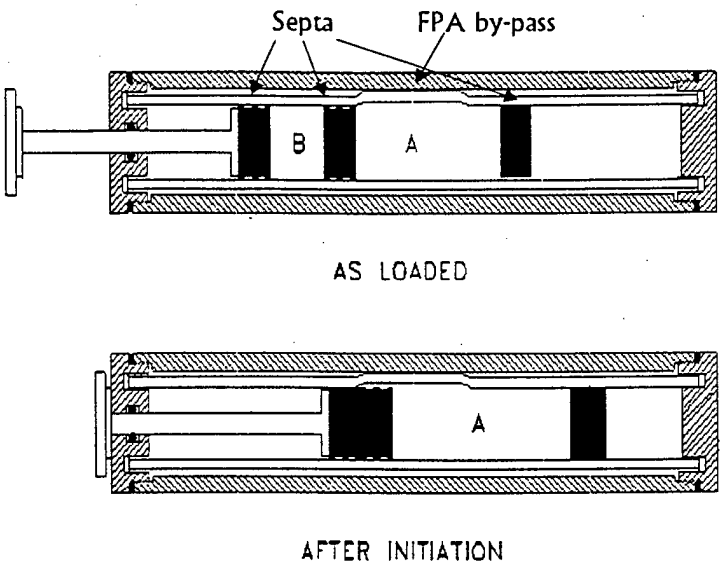

Figure 7-2 Fluids Processing Apparatus (FPA). The FPA contained an inner glass barrel housed in a Lexan outer sheath. Prior to launch, chamber A contained 3.5 ml of sterile growth medium, and chamber B held 0.5 ml of inoculum. Inoculation occurred 2.5 hours after launch when the contents of chamber B were injected into chamber A via the by-pass in the glass barrel.

The FPAs were contained in a Lexan sheath, which provided a second level of containment. A third level of containment was provided by an automated group activation pack (auto-GAP), shown in Figure 7-3. This container housed eight FPAs and provided the automatic activation function. The auto-GAP was placed in a generic bioprocessing apparatus/isothermal containment module (GBA-ICM). This fit in the shuttle middeck-locker insert and provided temperature control of roughly 24°C (Figure 7-3). The temperature for the flight and ground controls was automatically recorded every three minutes using a HOBO by Onset Computer Corporation.

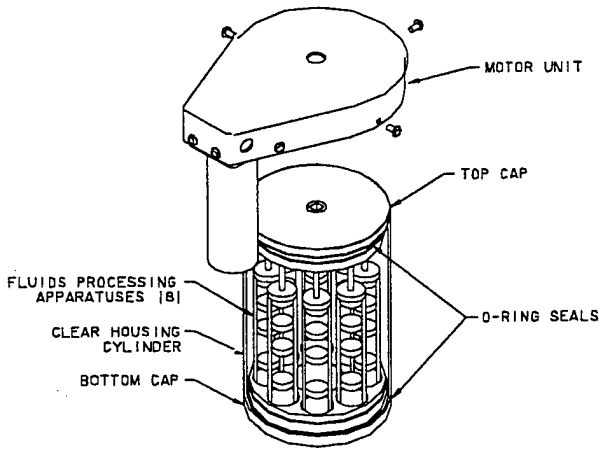

Figure 7-3 Automated Group Activation Pack (auto-GAP). The auto-GAP held 8 FPAs, four of which were used for this experiment. It automatically initiated the samples using the attached DC motor.

A total of four flight and four ground-control FPAs were used. To minimize the possibility of introducing variables unrelated to space flight, these eight samples underwent the same handling and loading procedures. This included shipping them to Kennedy Space Center together at 4°C. The samples were separated approximately 40 hours before launch, when the flight samples were loaded on the shuttle. The ground controls were then placed in an incubator at 24°C.

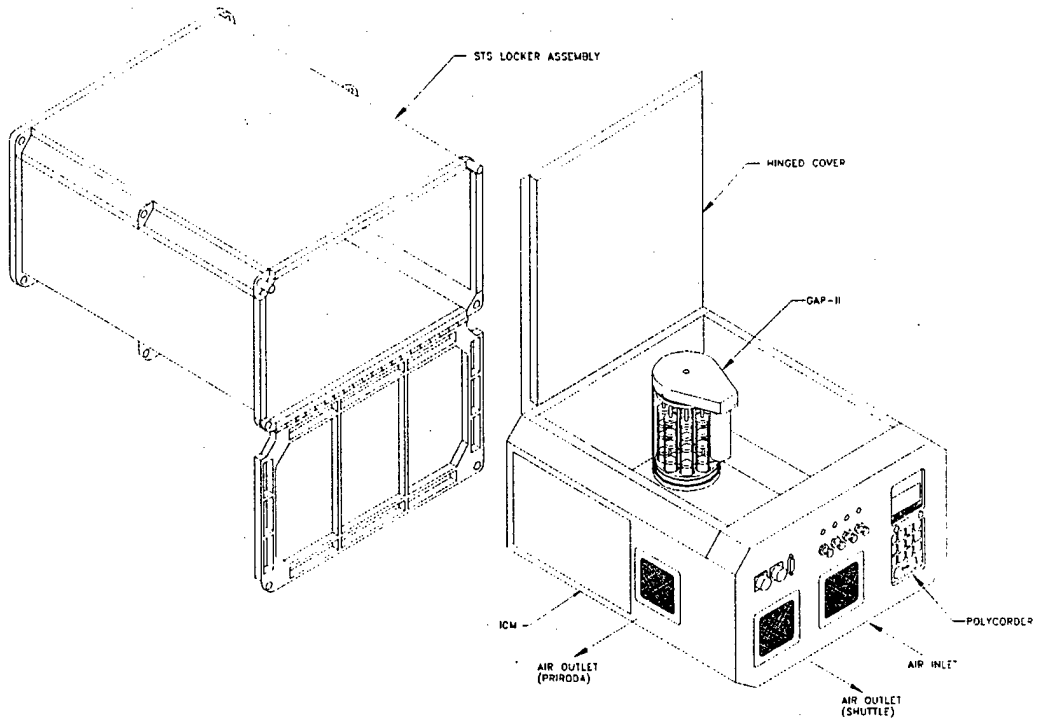

Figure 7-4 Generic Bioprocessing Apparatus - Isothermal Containment Module (GBA-ICM). The GBA-ICM provides temperature controlled stowage and autonomous in-flight processing for 9 auto-GAPs. The air outlet can be reconfigured to conform to vent to the side for Mir stowage or to the front when in the orbiter.

Liftoff occurred on October 29, 1998 at 2:18 PM EST. Two and a half hours after launch, the flight and ground-control samples were inoculated. The shuttle landed nine days later at 2:00 PM EST on November 7. Approximately five hours after landing the flight and ground-control samples were placed together in a refrigerator at 4°C. They were returned overnight to Boulder, Colorado, where the cell densities were measured, and the bacteria removed. As with the ground studies, the supernatant was then shipped with solid CO_2 to Bristol-Myers Squib for blind glucose analysis.

7.5 RESULTS

7.5.1 Ground Experiments

The bacterial growth curves for both ground experiments were consistent with the results from similar experiments discussed in Chapter 5. On average both clinostat experiments had a shorter lag phase and a higher final cell density than in the 1 *g* controls. The samples at 50 *g* had a much shorter lag phase and a lower cell final cell density than either experiments on the clinostat or the 1 *g* controls. These differences were all statistically significant. Details of these growth experiments are summarized in Table 5-1 on page 86. (The two experiments discussed in this chapter are numbered 9 and 10 in Table 5-1, and will be referred to by the same numbers here.)

Figure 7-5 shows the logistic bacterial growth curves for each acceleration condition during the first metabolism experiment (Experiment 9). The figure also shows the concentration of glucose remaining in the samples at various times during growth. Figure 7-6 shows similar data for the second metabolism experiment (Experiment 10). For all six growth curves, the glucose concentration did not decrease significantly until the exponential phase of growth. The plots also indicate that bacteria continued to consume glucose after reaching the stationary phase.

More importantly, both experiments supported the proposed hypothesis. The data indicated that "functional weightlessness" allows bacteria to metabolize glucose more efficiently than comparable 1 *g* controls. The last samples collected from the clinostat during Experiment 9 had 12% more new cells than the 1 *g* controls (shown in

Figure 7-5 A and B). However, they only consumed 6% more glucose. The last clinostat samples from Experiment 10 averaged 9% more cells and consumed the same amount of glucose as the controls (shown in Figure 7-6 A and B). Both experiments also indicated that *E. coli* metabolize glucose less efficiently at 50 *g* than at 1 *g*. The centrifuged samples from Experiment 9 averaged a 32% lower final cell density than the controls, while consuming only 6% less glucose. The last samples collected from the centrifuge during Experiment 10 had a 40% lower final cell density than the 1 g controls, yet consumed the same amount of glucose.

For both experiments the ratio of new cell mass per mass of glucose consumed (biomass yield) was compared for every sample point after approximately 49 hours of growth. This included the last portion of the exponential phase and all of the stationary phase. Prior to this point it was difficult to determine if any glucose had been consumed; as a result, the biomass yield was meaningless for data points with less than approximately 49 hours of growth. There were two sample points after 49 hours of growth during Experiment 9, and five during Experiment 10. The biomass yield for these seven sample times are shown for each inertial condition in Figure 7-7. This figure also includes the *n* value, the average change relative to the 1 *g* controls, and the *p* value for all seven sample times.

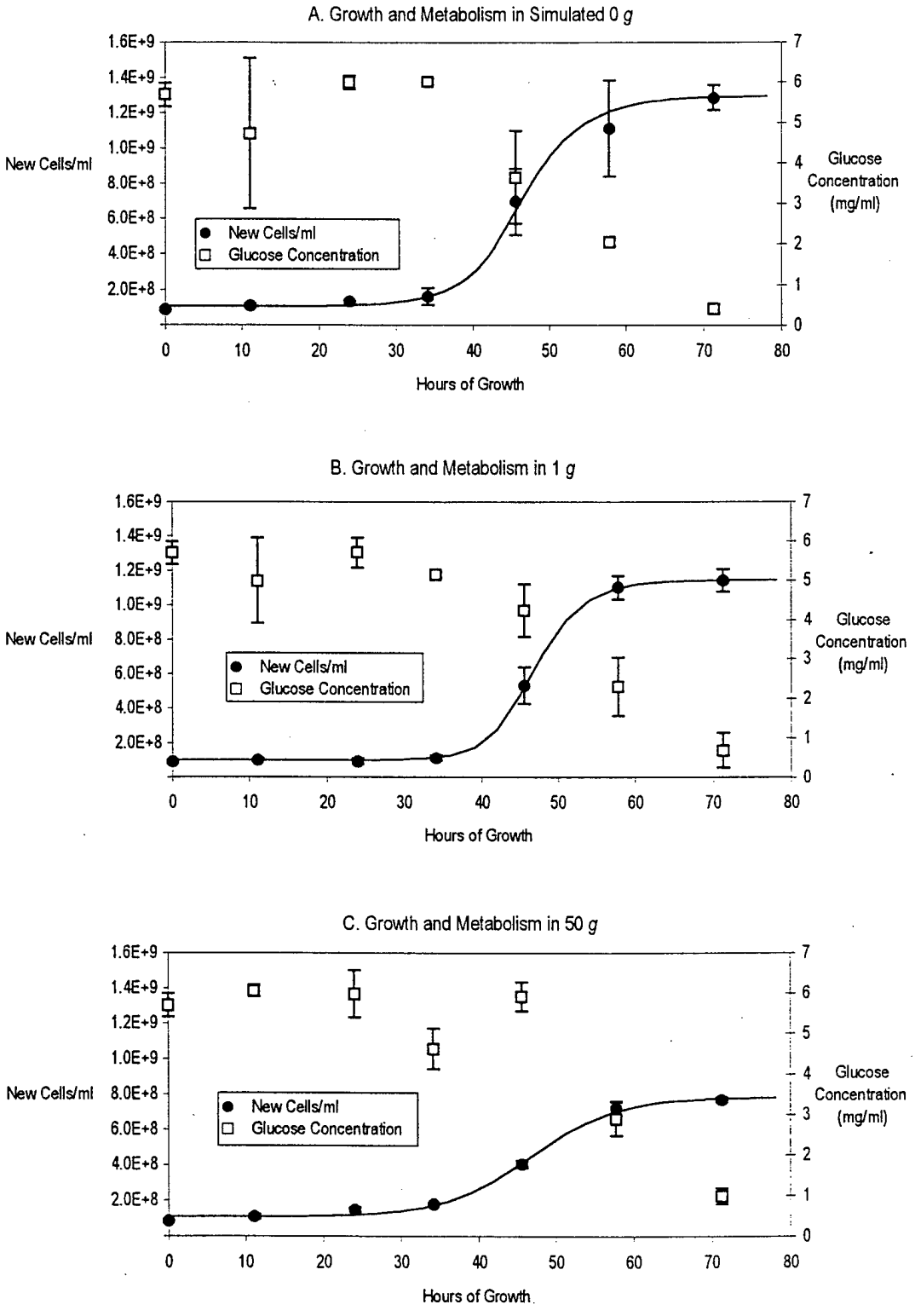

Figure 7-5 Growth and Metabolism of *E. coli* during (A) Clinorotation, (B) 1 *g*, and (C) 50 *g* for Ground Experiment 9

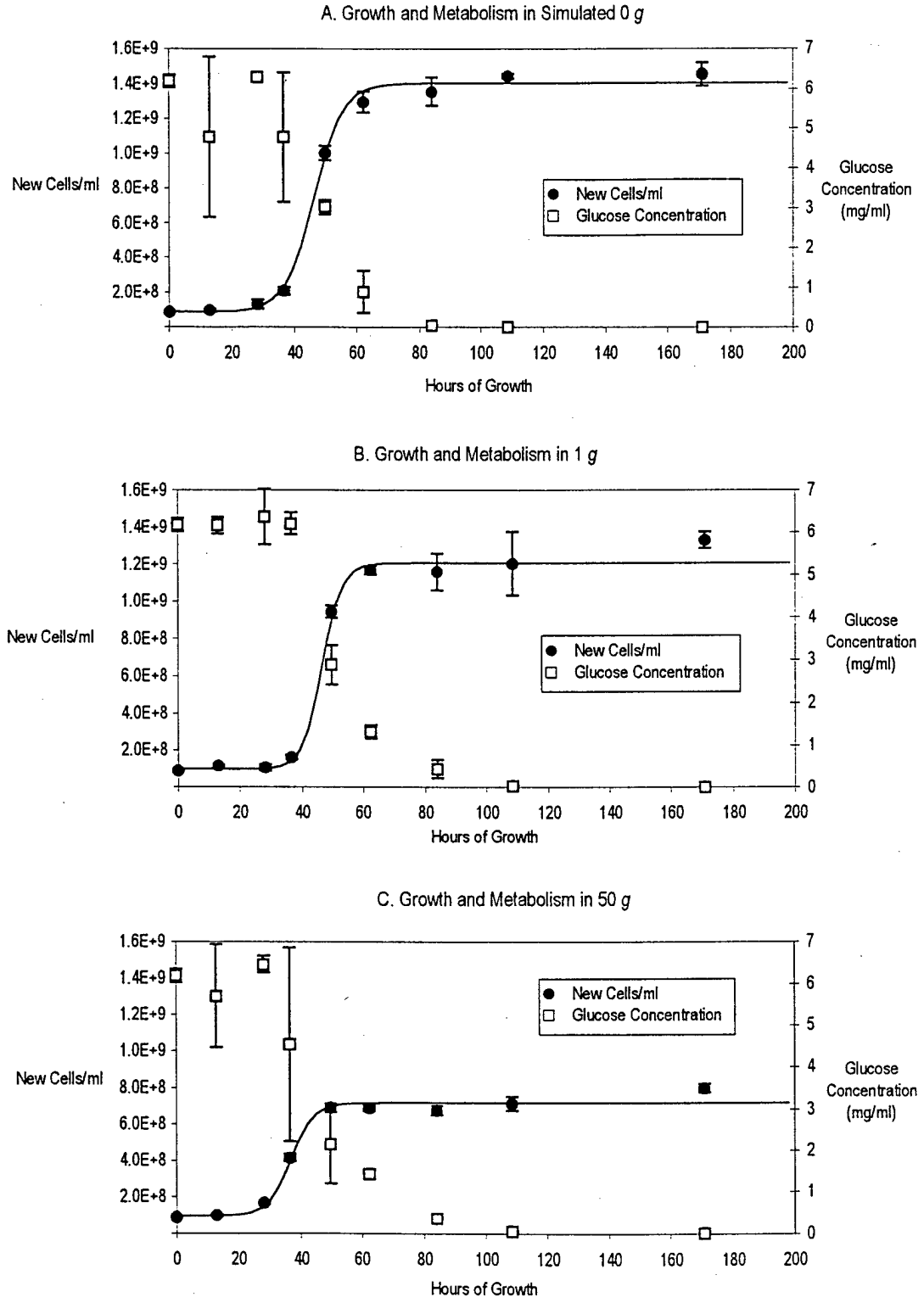

Figure 7-6 Growth and Metabolism of *E. coli* during (A) Clinorotation, (B) 1 *g*, and (C) 50 *g* for Ground Experiment 10

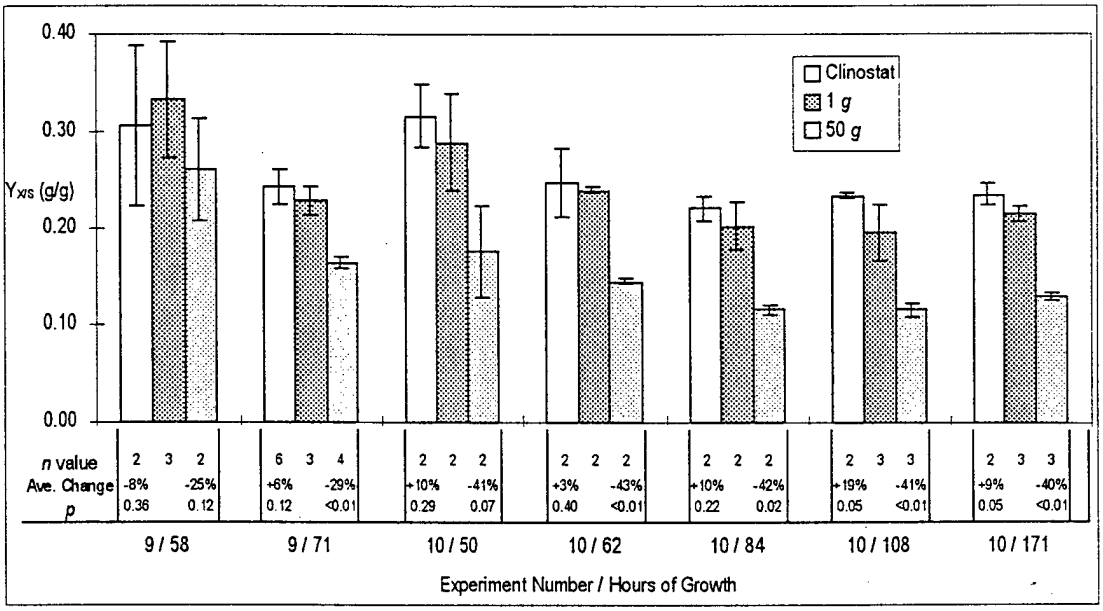

Figure 7-7 Biomass Yield, $Y_{X/S}$, (Mass of New Cells per Mass of Glucose Consumed) for Ground Experiments 9 and 10. On average the samples on the clinostat had a 7.3% higher biomass yield than their paired 1 *g* controls ($p = 0.035$). The samples on the centrifuge averaged a 35.5% lower biomass yield than the controls ($p < 0.01$).

For the seven different sample times (two from Experiment 9 and five from Experiment 10), the clinostat samples had the highest average biomass yield for six different sampling times. Relative to the 1 *g* controls, two of these differences were statistically significant ($p < 0.05$), and one indicated a trend ($0.05 < p < 0.15$). The results were summarized by comparing all of the paired data from the same sampling time. This showed the average clinostat sample had a 7.3% higher biomass yield than in comparable 1 *g* controls. This difference was statistically significant ($p = 0.035$).

The average biomass yield for the samples at 50 *g* was consistently lower than that of the 1 *g* controls. For all seven different sample times, the 50 *g* samples had a lower average biomass yield when compared with the control samples. Five of these

comparisons were statistically significant ($p < 0.05$), and the other two indicated a trend ($0.05 < p < 0.15$). On average the 50 g samples had a 35.5% lower biomass yield than the 1 g controls, which was statistically significant ($p < 0.01$).

7.5.2 Flight Experiment

On average the four samples flown on STS-95 had 25.4% more cells than the four ground controls. This difference was statistically significant ($p < 0.01$). Also, in all eight samples the bacteria consumed all of the glucose. Therefore, the space samples had a 25.4% higher biomass yield than the ground controls.

The ground controls were maintained close to the desired temperature of 24° C, averaging 23.3° C during the nine-day experiment. However, the temperature of the flight samples fluctuated considerably (see Figure 7-8) during this same time. The temperature of the flight samples averaged 26.6° C between inoculation and landing, and at one time briefly dropped to 12° C. Although these temperature fluctuations could affect bacterial growth, post-flight analysis indicated that is doubtful that temperature fluctuations significant affected the flight samples' final cell density or their final glucose concentration.

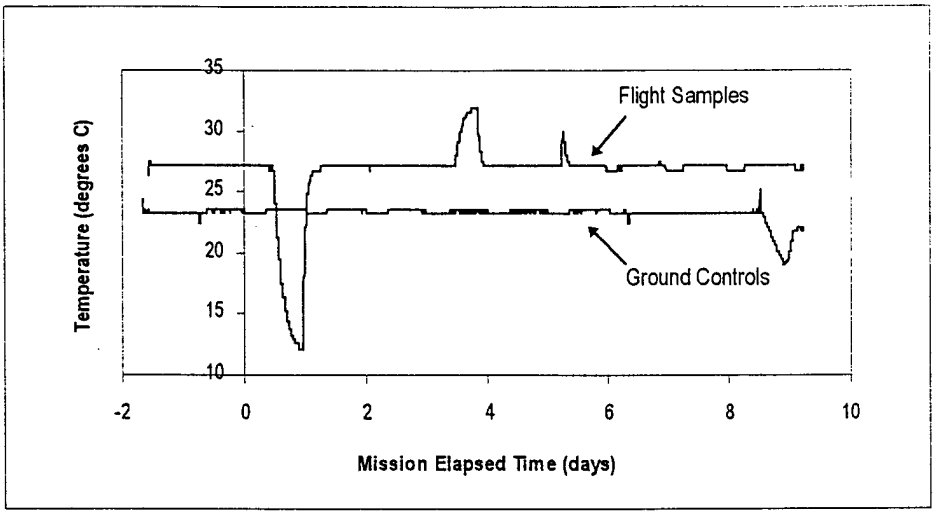

Figure 7-8 Temperature of the Flight Samples and Ground Controls. The temperature of the ground controls was approximately constant, averaging 23.3° C. However, the temperature of the flight samples fluctuated considerably, averaging 26.6° C.

The unexpected temperature fluctuations on orbit most likely did not bias the final cell density. The finding that space flight resulted in a significantly higher final cell density compared with 1 *g* controls is consistent with almost all previous research (Ciferri *et al.*, 1986; Klaus *et al.*, 1997, 1994; Klaus, 1994; Kordium *et al.*, 1980; Mattoni, 1968; Mennigmann and Lange, 1986; Pierson *et al.*, 1993). Also, in many ground experiments it was found that temperature changes between 20° C and 30° C do not affect *E. coli's* final cell density. Ground studies found no difference in the final cell density for *E. coli* cultures grown at a constant 21, 24.5, or 30° C. The only noticeable effect occurred in samples held at 18.5° C. These samples averaged a 5% lower final cell density than controls held at 24.5° C. Similar experiments found no difference in the final cell density of samples grown at 20° C or 24° C. In addition,

post-flight ground studies showed no difference in the final cell density for ground samples held at 24° C and similar samples with the same temperature profile from STS-95 through day 3.5. This profile included the largest temperature deviation to approximately 14° C (shown in Figure 7-9, line B). All of these results indicate that if the temperature remains between roughly 20° C and 30° C for majority of the experiment, final cell density is not affected. It is therefore reasonable to conclude that the temperature deviations on STS-95 did not affect the final cell density.

It is also doubtful that the temperature changes affected the amount of glucose consumed on orbit. Ground studies consistently indicated that *E. coli* consume all of the glucose in the medium provided the temperature remains predominantly between 20° C and 26° C. For example, Experiment 10, shown in Figure 7-6, had a total of 16 samples collected after 4 days. All the glucose had been consumed from all 16 of these samples. Four post-flight temperature studies also supported these findings. These experiments included 29 additional samples collected after nine-day experiments using four different temperature profiles. These four temperature profiles, which are shown in Figure 7-9, included:

A. Temperature maintained at approximately 24° C.

B. A temperature profile similar to that of the flight samples through day 3.5, averaging 25.1° C during the nine-day experiment.

C. A temperature profile similar to that in flight during the entire nine days, averaging 25.2° C (1.4° C lower than the flight samples).

D. A profile including many fluctuations between 16° C and 19° C, with an average of 18.6° C.

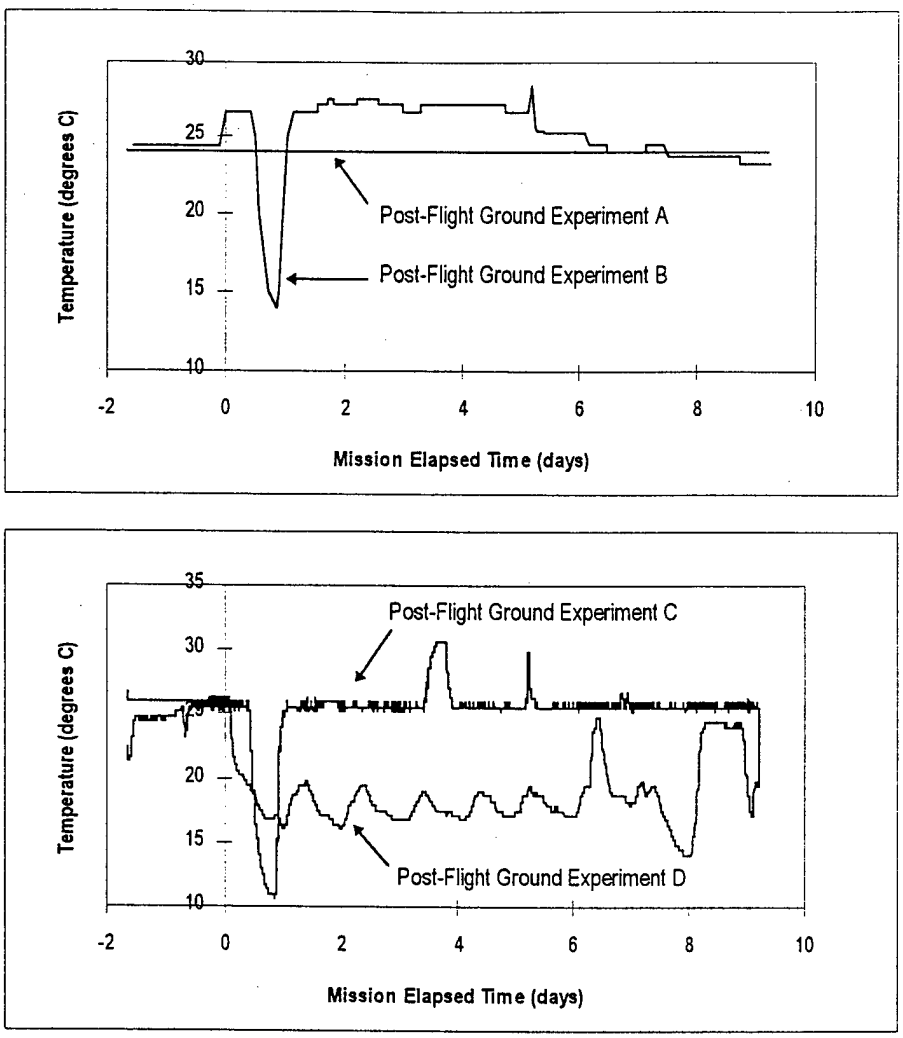

Figure 7-9 Temperature Profile for Post-Flight Ground Studies. To determine any effects from the temperature fluctuations on orbit, four ground studies were completed. These temperature profiles included: (A) Approximately 24°C (B) Attempt at reproducing the first portion of the flight temperature profile, (C) Reproducing the entire flight temperature, and (D) A much colder profile, averaging 18.6°C. These correspond to the four ground experiments shown in Table 7-1.

All of the glucose was consumed from every sample during the temperature profile in Experiments A and B. Of the eight samples collected from Experiment C, only one had a detectable level of glucose remaining (0.17 g/l), and the average level

was only 0.02 g/l. Only the samples from Experiment D consistently showed some glucose remaining. These samples, which had a medium temperature of 18.6° C, had an average of 0.30 g/l of remaining glucose. This meant the bacteria consumed 4.4% less glucose than the other post-flight experiments. Unfortunately, due to thermal control problems these four temperature profiles did not have suitable controls, so comparisons of yields were not possible. However, these findings, which are summarized in Table 7-1, do indicate that as long as the temperature remains predominantly between 20° C and 26° C, the medium is glucose limited. Therefore, the temperature fluctuations on orbit most likely did not affect the final glucose concentration.

Table 7-1 Final Glucose Concentrations from Space Flight, Ground Controls, and Four Post-flight Experiments. Four ground experiments, with the temperature profiles shown in Figure 7-9 indicated that the medium is glucose limited provided the average temperature remains above approximately 20° C.

	Ave. Temp. (deg. C)	*n* value	Average Final Glucose Concentration (g/l)
STS-95 Flight Experiment	26.6	4	0.00
STS-95 Ground Controls	23.3	4	0.00
Ground Experiment A	~24*	9	0.00
Ground Experiment B	25.1	8	0.00
Ground Experiment C	25.2	8	0.02
Ground Experiment D	18.6	4	0.30

* The average temperature for Ground Experiment A was estimated using measurements every 8 to 10 hours. The temperatures for the other experiments were more accurately determined using a HOBO by Onset Computer Corporation that automatically recorded the temperature every three minutes.

7.6 DISCUSSION AND CONCLUSIONS

The experiments discussed in this chapter support the proposed hypothesis that *E. coli's* biomass yield (mass of new cells normalized per mass of glucose consumed) is inversely related to inertial acceleration. When cultivated at 50 *g*, the average *E. coli* sample was 35.5% less efficient at metabolizing glucose than similar experiments in 1 *g* ($p < 0.01$). Experiments simulating weightlessness with a clinostat had an average biomass yield that was 7.3% higher than in comparable 1 *g* controls ($p = 0.035$). Also, experiments on orbit averaged a 25.4% higher biomass yield than in controls ($p < 0.01$). Although the space samples did not have the same temperature profile as the ground controls, post-flight experiments indicated that this did not affect the results.

These findings support the proposed model that bacterial growth creates buoyancy-driven convection of the excreted by-products. As bacteria grow they consume glucose from their surrounding medium. Data from previous chapters indicated that this decreases the solute concentration in the region surrounding each cell. This decreased solute concentration is believed to reduce the medium's density and cause the beneficial by-products to rise away from the cells. It is therefore proposed that at high accelerations buoyancy-driven convection causes bacterial metabolism to be less efficient than in 1 *g* controls. In contrast, experiments at hypogravity (both real and simulated) allow the excreted by-products to remain near the bacterial cells. This is believed to improve bacterial metabolism, allowing the cells to more efficiently convert glucose into new cells.

The experiments discussed in this chapter support this theory. However, additional research in this area is needed. Future experiments with *E. coli* should not only measure the cell density and amount of nutrients consumed; they should also determine the concentration of by-products such as acetic acid, ethanol, and formic acid. Although *E. coli* is an excellent organism for initial studies, similar experiments should also be accomplished with bacteria that are more important economically. *Streptomyces* is a good example. There has been one space-flight experiment that found *Streptomyces* produced more antibiotics on orbit (Lam *et al.*, 1999). However, it still is not known if they produce more antibiotics per unit of nutrient, or if clinorotation achieves similar results. Further investigations in this area are needed.

CHAPTER 8

POTENTIAL INDUSTRIAL APPLICATIONS

8.1 INTRODUCTION

This research has focused on quantifying the effects of acceleration on bacterial growth and metabolism, and identifying a physical mechanism that could explain the observed results. This may eventually lead to improved terrestrial pharmaceutical production or space-based bioprocessing. This chapter explores the potential economic benefits of these two industrial applications.

8.2 BACKGROUND

As detailed in Chapter 2, many previous experiments have found that microgravity stimulates the growth of bacteria, resulting in a shorter lag phase and higher final cell density than in similar experiments on Earth. New research, detailed in the previous chapter, also suggested that bacteria use nutrients more efficiently in space. Other experiments have previously reported that microorganisms produce more antibiotics on orbit. In one experiment, *Streptomyces* had a 115% higher specific productivity of actinomycin D in space than in comparable ground controls (Lam *et*

al., 1999). Another experiment found the fungus *H. fuscoatra* produced 190% more antibiotics on orbit (Lam *et al.*, 1998). Although these are impressive changes, these initial experiments were performed in FPAs or similar containers, which did not provide optimal fermentation conditions. As a result, the yields on orbit, although higher than in similar ground controls, were lower than yields associated with optimized terrestrial pharmaceutical production. Therefore, future experiments on orbit will use a bioprocessing device currently being developed that will provide optimized fermentation conditions.

These findings are not only interesting scientifically. There is also a potential economic benefit. If it can be determined why these changes occur on orbit, the knowledge gained could possibly be used to improve terrestrial pharmaceutical production. It is also plausible that space-based bioprocessing will someday become economically feasible for certain high-value products.

The feasibility of commercial space-based bioprocessing has been the focus of a number of NASA conferences and reports (Milov *et al.*, 1989; McDonnell Douglas, 1978a; McDonnell Douglas, 1978b; Mayeux, 1976). These studies have explored such things as the involvement of industrial companies, required laboratory equipment, and design considerations for space-based bioreactors. Small bioreactors have also been built and tested on orbit (Villeneuve and Dunlop, 1992; Walther *et al.*, 1994, 1996a, 1996b). However, these reports contained very little economic analysis and did not discuss the potential benefit associated with transferring knowledge gained in space to terrestrial bioprocessing. This chapter addresses both of these issues.

8.3 IMPROVED TERRESTRIAL PRODUCTION

The production of antibiotics by microorganisms account for a large portion of the pharmaceutical industry. The annual global market for antibiotic production is in excess of $20 billion (Klaus *et al.*, 1998a). In 1996, one pharmaceutical company reported a $2.9 billion profit from pharmaceutics, with the majority of this stemming from antibiotic production (Bristol-Myers Squibb, 1996).

Microoganisms and animal cells are used to produce many drugs other than antibiotics. Compactin, Bristol-Myers Squibb's number one selling drug for controlling cholesterol, is produced from a secondary metabolite of a fungus (Klaus *et al.*, 1998a). Bristol-Myers Squibb will also soon begin producing TAXOL in large fermentation tanks, similar to the way penicillin is produced (Bristol-Myers Squibb, 1996). This new cancer fighting drug had sales of $813 million in 1996 (Bristol-Myers Squibb, 1996). Another profitable drug, Epogen, is produced from animal-cell cultures by Amgen. This drug, used for treating anemia, is used by over 220,0000 patients in the United States and had sales in 1997 of $1,161 million (Amgen, 1997).

Considering the size and market of this large industry, even a small improvement in production efficiency would equate to a substantial increase in profit. Therefore, a considerable amount of research has explored methods of increasing bioprocessing efficiency. Almost all of this research has focused on improving nutrient availability (Harcum *et al.*, 1992; Yee and Blanch, 1992; Lischke *et al.*, 1993; Shiloach *et al.*, 1996; Robbins and Taylor, 1989; Luli and Strohl, 1990) or genetically altering the microorganisms (Gram, 1994; Dedhia *et al.*, 1994; Shiloach *et al.*, 1996; Ciferri *et*

al., 1986). In contrast, the data presented in the last chapter indicated that varying inertial acceleration may provide a new method of increasing pharmaceutical production efficiency.

If the knowledge gained from research in space can be transferred to terrestrial fermentation processes, it could result in a significant profit increase. For example, suppose a new bioreactor, which might be similar to a large clinostat, allowed just a 1% increase in the efficiency of terrestrial fermentation. Making some reasonable assumptions, this would equate to an annual profit increase for Bristol-Myers Squibb of over $6 million a year (calculations are shown below).

Profit Calculations

The pharmaceutical segment of Bristol-Myers Squibb had sales of $8.7 billion in 1996, and their profit was $2.9 billion (Bristol-Myers Squibb, 1996). If we assume 25% of their profit involved fermentation, then their total cost from fermentation was

$$\text{Cost of fermentation} = 0.25(\$8.7 \text{ billion} - \$2.9 \text{ billion}) = \$1{,}450 \text{ million}. \quad (8\text{-}1)$$

If a new bioprocessing method produced the same yield using less raw materials, the variable costs associated with fermentation would be reduced. This would include the costs for utilities, raw materials, and labor associated with fermentation. All other costs would remain the same (labor, raw materials, and utilities associated with non-fermentation processes, taxes, depreciation, etc.). Using a detailed example from Bailey and Ollis (1986), it was estimated that 46% of industrial fermentation processes are variable costs associated with fermentation, while the

remaining 54% are fixed costs. Using this figure, Bristol-Myers Squibb's annual variable cost associated with fermentation is:

$$\text{Variable cost of fermentation} = 0.46(\$1{,}450 \text{ million}) = \$667 \text{ million.} \quad (8\text{-}2)$$

A 1% increase in efficiency would reduce this cost by 0.99% (100/1.01=99.0099%). This cost savings would therefore equate to an increase in annual profit of approximately

$$\text{Annual Profit Increase} = 0.0099(\$667 \text{ million}) = \$6.6 \text{ million.} \quad (8\text{-}3)$$

8.4 SPACE-BASED BIOPROCESSING

It is also possible that the knowledge gained from space research might not be easily transferred to terrestrial bioprocessing. Some drugs may only have high yields on orbit. Suppose, for example, that space flight allowed a drug to be produced with a 100% higher yield than terrestrial bioprocessing. Would it be economical to produce this drug on orbit? More specifically, under what conditions would the higher yield on orbit offset the additional costs required for space-based bioprocessing? Stated mathematically, when will the following condition hold true?

$$\text{Added Economic Value} > \text{Added Cost of Space Access} \quad (8\text{-}4)$$

To quantify the additional economic value and cost associated with space-based bioprocessing, the following assumptions were made.

1. Space bioprocessing increases the specific yield of a product (mass of product per mass of substrate) by 100% relative to terrestrial pharmaceutical production.

2. Pharmaceutics will be produced in a small bioreactor on the International Space Station. The reactor will be automated, and not require any astronaut time (which would cost approximately $10/sec) (Elliott, 1994).

3. The major costs associated with space-based bioprocessing, which are not involved with terrestrial bioprocessing, are:
   a. Research and development of the space-based bioreactor.
   b. One time transport of the bioreactor to space.
   c. Periodic transport of fresh substrate for bioprocessing on orbit.
   It is assumed that over the life of the project the cost of each of these is roughly equivalent (Elliott, 1994). Therefore, for simplicity, the total additional cost required for space-based fermentation is three times the cost required to transport the nutrient to orbit.

4. The substrate has the density of water (1 g/ml).

Using these assumptions, Equation 8-4, is

$$\Delta M_P\left(V_P\right) > 3M_S\left(C_L\right) \tag{8-5}$$

where

ΔM_P is the additional mass of the product produced on orbit compared with terrestrial production.

V_P is the value of the product per unit mass.

M_S is the mass of the substrate required to yield ΔM_P.

C_L is the cost to launch the substrate per unit mass.

C_L is the easiest of these terms to estimate. The cost to launch something into Low Earth Orbit (LEO) on the Shuttle is approximately $10,000/kg (Wong *et al.* 1995). To provide a better understanding of these costs, consider that the price of gold is currently about $300 per ounce, or $10,600/kg. In other words, to carry something into orbit, requires its weight in gold. Although this is very expensive, some pharmaceuticals are a thousand times more valuable than gold (Bailey and Ollis, 1986).

We can now substitute the value for C_L into Equation 8-5, and also divide both sides by M_S.

$$\frac{\Delta M_P}{M_S}\left(V_P\right) > 3\left(\$10{,}000 / \text{kg}\right) \tag{8-6}$$

This ratio, $\Delta M_P/M_S$, is simply the change in specific yield of product per unit substrate, or $\Delta Y_{P/S}$. Because yields are commonly shown in units of mg/l, and the substrate's density was assumed to be 1 g/ml, Equation 8-6 can be modified slightly to

$$\Delta Y_{P/S}\left(V_P\right) > 30{,}000 \tag{8-7}$$

where $\Delta Y_{P/S}$ is in units of mg/l, and V_P, is in units of $millions/kg.

Because the yield on orbit was assumed to be 100% greater than terrestrial bioprocessing, $\Delta Y_{P/S}$ is equivalent to $Y_{P/S}$ from terrestrial fermentation. Typical values for $Y_{P/S}$ vary depending upon the product, the microorganism, the substrate, and the efficiency of the bioreactor. Because a bioreactor on the Space Station will have to be relatively small, a reasonable estimate for $\Delta Y_{P/S}$ can be made by referencing typical yields from a small bioreactor. One such reactor, the High Aspect Rotating Vessel

(HARV), was discussed in Chapter 2. This reactor uses only a 50 ml or 100 ml culture chamber. The first three columns of Table 8-1 summarize typical yields from this reactor for different microorganisms and secondary metabolites (Fang *et al.*, 1997a, 1997b, 1997c). The values in the last column of Table 8-1 were calculated using Equation 8-7. This column shows the estimated minimum required product value for a similar operation to be economically profitable in space.

Table 8-1 Product Yields from a Small Bioreactor and the Required Product Value for Economically Profitable Space-Based Bioprocessing

Microorganism	Product	$Y_{P/S}$ (mg/l)*	Minimum Required V_P ($millions/kg)
Streptomyces clavuligerus	antibiotic, β-lactam	3	10,000
Bacillus brevis	antibiotic, gramicidin S	1,000	30
E. coli	antibacterial polypeptide, microcin B17	100	300

* Yields from Fang *et al.* (1997a, 1997b, 1997c), respectively.

Referencing the far right column of Table 8-1, the estimated minimum product value for profitable space-based bioprocessing ranges from $30 million/kg to $10 billion/kg. It is, therefore, very doubtful that space-based bioprocessing will be profitable for a majority of pharmaceutics. However, considering the cost of some pharmaceutics is greater than $10 million/kg (Bailey and Ollis, 1986), space-based bioprocessing could someday be economically feasible for a few drugs. For example, only a few kilograms of Amgen's drug Epogen costs roughly 500 to 1,000 million. If a drug such as this could be produced on orbit with a much higher yield compared with terrestrial bioprocessing, it would likely be economically profitable.

8.5 CONCLUSIONS

The data in Chapter 7 showed that varying inertial acceleration may provide a new method of increasing pharmaceutical production efficiency. While these findings are only preliminary, they indicate the possibility of future economic gain. The simple analysis in this chapter suggests that space-based bioprocessing may become economically profitable for a few drugs. Assuming launch costs do not change, it would require a high yield of a very valuable product on orbit, costing between $10 million and $100 million per kg. A more likely benefit of space-based fermentation research is the transfer of knowledge to terrestrial bioprocessing. If this allows just a 1% higher specific yield from terrestrial fermentation, it would increase the annual profit of a pharmaceutical company like Bristol-Myers Squibb by approximately $6 million. Due to these substantial economic benefits, further research is being conducted to quantify the effects of microgravity on secondary metabolism.

CHAPTER 9

CONCLUSIONS

In summary, the results of experimental research in this dissertation support the primary hypothesis outlined in Chapter 1:

> *Inertial acceleration affects the growth and metabolism of suspended E. coli cultures by altering the transport phenomena in the external fluid environment of the cells.*

This hypothesis was tested by five sub-hypotheses. Data supported all of these sub-hypotheses, which were formulated based upon a new model of bacterial growth. This chapter begins by reviewing this model, and explaining how it was used to predict the effects of acceleration on *E. coli's* growth and metabolism. The conclusions of each chapter are then reviewed, followed by recommendations for additional research.

9.1 PROPOSED MODEL

In chapter 4 a new physical model was proposed to explain how acceleration affects bacterial growth and metabolism. This model claims that buoyancy-driven convection is the dominant gravity-dependent force that separates bacterial by-products from their cells. As bacteria consume glucose and excrete their by-products, they decrease the solute concentration in their surrounding medium. This density difference is believed to create natural convection currents around metabolizing

bacteria, which quickly separate by-products from their cells. Some of these by-products, such as carbon dioxide, have been shown to be beneficial for reducing the lag phase (Barford *et al.*, 1982; Gottschalk, 1986). Other by-products, such as cofactors or enzymes, would also be beneficial to growth.

This model was used to explain the observed effects of microgravity on bacterial growth and also to accurately predict the effects of microgravity on bacterial metabolism. In microgravity, both real and simulated, reduced effects of convection allow excreted by-products to remain near their cells. This results in a shorter lag phase, a higher final cell density, and more efficient consumption of nutrients (higher biomass yield) compared with 1 *g* controls. Microgravity also allows the bacteria to remain colloidal. This is believed to improve their nutrient availability, which contributes to the observed higher final cell density relative to 1 *g* controls.

The proposed model was also used to correctly predict the effects of hypergravity on final cell density. At high levels of acceleration, critical by-products are quickly separated from the cells due to buoyancy-driven convection. Because some of these by-products are needed for cellular growth, the final cell density was expected to decrease as acceleration increased. The final cell density was also expected to decrease because the bacteria would become stacked on top of each other very quickly, resulting in a lower final cell density as acceleration increases.

Hypergravity was expected to have a non-monotonic effect on the length of the lag phase. At accelerations between approximately 10 *g* and 100 *g*, sedimentation quickly concentrates the bacteria at the bottom of their culture tube. This is believed

to initially be beneficial for growth because the bacteria are surrounded by the by-products excreted from many cells. As a result their lag phase is significantly reduced relative to 1 *g* controls or experiments at hypogravity. However, for higher levels of acceleration, above approximately 100 *g*, the effects of buoyancy-driven convection begin to outweigh the initial benefits from sedimentation. The bacteria still become concentrated at the bottom of the culture tube very quickly, but the buoyancy forces have a greater effect as acceleration increases. This is believed to separate the by-products from their cells at the bottom of the culture container, causing the lag phase to increase at higher levels of acceleration. Eventually this results in no difference in the length of the lag phase relative to 1 *g* controls.

The proposed model also correctly predicted the effects of hypergravity on *E. coli's* metabolism. Because buoyancy-driven convection increases with higher levels of acceleration, it was hypothesized that hypergravity would reduce *E. coli's* biomass yield. At high levels of acceleration beneficial by-products, which might include cofactors and enzymes, are quickly carried away from the cells by buoyancy-driven convection. Therefore, the cells were not expected to metabolize glucose as efficiently as similar controls at 1 *g*, and the ratio of new cell mass per mass of glucose consumed was expected to decrease for higher levels of acceleration.

9.2 SUMMARY OF RESEARCH

Chapter 2 provided a comprehensive literature review. This was followed by a discussion of the general materials and methods in Chapter 3. The remaining chapters

detailed the chain of research results. The conclusions from these chapters are summarized below:

9.2.1 Chapter 4

In chapter 4 the theory that sedimentation alone can explain the observed shortened lag phase on orbit was investigated. Two experiments found that preconditioned medium does reduce the lag phase for suspended *E. coli* cultures. This supported the idea that higher concentrations of excreted by-products surrounding bacteria reduce the lag phase. However, computer simulations indicated that the absence of sedimentation on orbit does not change the concentration of by-products surrounding bacteria in space. It was therefore proposed that an additional gravity-dependent process other than sedimentation also affects bacterial growth.

A review of the crystal-growth literature revealed another possible gravity-dependent process. As a crystal grows the solute concentration in the immediate surrounding medium decreases. This decreased density creates small convection currents around the crystal. On orbit, however, convection currents do not occur, which allows crystals to achieve larger and more uniform growth (Brailovskaya *et al.*, 1994; Fehribach and Rosenberger, 1989; McCay and McCay, 1994; Pusey and Naumann, 1986; Pusey *et al.*, 1988, 1986). **This experimental and conceptual study led to the proposed model of buoyancy-driven convection associated with bacterial growth.**

9.2.2 Chapter 5

Nineteen ground experiments were conducted to test the proposed model over a broad range of accelerations. A clinostat was used to simulate 0 *g*; a novel method of simulating 0.5 *g* was achieved by inclining a clinostat; and a centrifuge was used to achieve 50 *g*, 180 *g*, and 400 *g*. All nineteen experiments supported the four sub-hypotheses concerning the effects of acceleration on final cell density and the length of the lag phase. **Final cell density was inversely related to the level of acceleration, and acceleration affected the lag phase in a non-monotonic manner, as predicted.** Simulations of hypogravity also had a slower growth rate and a slightly longer exponential phase than 1 *g* controls. **All of these results can be explained by the proposed model of buoyancy-driven convection in the medium surrounding metabolizing bacteria.**

9.2.3 Chapter 6

Chapter 6 continued investigating the possibility of buoyant plumes rising from bacteria. This included **a photograph of a plume rising from many bacterial cells** clustered on the bottom of a culture tube. This plume was analyzed mathematically. Its velocity, Reynolds number, and boundary layer were estimated. **All of these calculations agreed with observed results.** The same equations were then used to estimate the plume velocity from one bacterium. This showed that **the initial plume velocity from a single cell is about 33 times greater than the *E. coli* sedimentation rate.** This supported the hypothesis that buoyancy-driven convection is the dominant

gravity-dependent force that separates excreted by-products from their cells, not sedimentation. Additional analysis showed that despite the relatively high plume velocity, clinorotation can still reasonably simulate the quiescent fluid environment achieved in space. Further analysis suggested that for accelerations above 10 to 50 g, final cell density is inversely proportional to plume velocity, and the length of the lag phase is directly proportional to plume velocity. **Therefore, above 10 to 50 g, final cell density is a function of $g^{-1/2}$, and the length of the lag phase is a function of $g^{1/2}$.**

9.2.4 Chapter 7

Two ground experiments, using a clinostat and centrifuge, and one space-flight experiment were conducted to determine the effects of acceleration on the metabolism of *E. coli*. These experiments supported the proposed hypothesis that **the biomass yield (mass of new cells normalized per mass of glucose consumed) is inversely related to acceleration.** Samples on the clinostat averaged a 7.3% higher biomass yield than in control samples ($p = 0.035$). Experiments at 50 g had a 35.5% lower yield on average than in controls ($p < 0.01$). The experiment on orbit had a 25.4% higher average biomass yield than comparable ground controls ($p < 0.01$). All of these results support the proposed theory that bacterial growth creates buoyancy-driven convection in the depletion zone surrounding each cell, and this results in a more efficient metabolism of nutrients at lower levels of acceleration.

9.2.5 Chapter 8

Two potential industrial applications of this research were addressed. Using simple analyses, it was estimated that space-based bioprocessing may only be economically profitable for a few select pharmaceutics. These drugs, however, will have to cost over $10 million per kg, and have a very high yield on orbit. A more likely benefit of space-based fermentation research is the transfer of knowledge to terrestrial bioprocessing. Using some conservative assumptions, it was estimated that **if the knowledge gained from space-based research can lead to an increase of 1% in the specific yield of terrestrial fermentation, it would increase the annual profit for a company like Bristol-Myers Squibb by over $6 million.**

9.3 AREAS OF FURTHER RESEARCH

A number of areas are suggested for further research. These include improving existing hardware, fermentation experiments, investigating the effects of acceleration on antibiotic effectiveness, crystal growth experiments, and analysis of forces at the cellular level. The research recommended for each of these areas is outlined below.

9.3.1 Improving Existing Hardware

The research in this dissertation began with the design and fabrication of a large clinostat, which was able to hold up to eight FPAs. This was a critical piece of hardware, allowing multiple samples to be cultivated at one time. However, the design of a new clinostat, with additional capabilities would be very helpful for further

research. Ideally a new clinostat would allow thermal control. An improved method of rotating the tubes, with less friction to prevent the drive belt from slipping would also be very beneficial.

9.3.2 Fermentation Experiments

There are many possible fermentation experiments that are worthy of future research. For example, the experiments outlined in Chapter 7 should be duplicated, particularly the flight experiment. If possible, these experiments should include samples collected at multiple time points on orbit, not just a single sample time. Larger *n* values and more frequent sampling from the ground experiments would also be beneficial. This would allow a better estimate of the rate of glucose consumption during the early portion of the exponential phase.

To determine the efficiency of metabolism at different levels of acceleration, the experiments discussed in Chapter 7 measured one input (mass of glucose consumed) and one output (mass of new cells). A logical "next step" is to repeat these experiments while measuring many outputs, including the production of acetic acid, ethanol, and formic acid. Based upon the proposed model, the specific yield of all of these by-products should decrease as acceleration increases. To assist anyone who takes this "next step" the frozen supernatant from the flight and ground-control samples has been saved.

Similar experiments should also be accomplished with bacteria that are more widely used by pharmaceutical companies. *Streptomyces* is an excellent example. Nearly 75% of all antibiotics currently available are isolated from *Streptomyces* (Atlas,

1997). Future experiments with *Streptomyces* should measure the amount of nutrients consumed as well as the amount of by-products produced on orbit. It would also be worthwhile to accomplish this with ground studies using a clinostat and centrifuge. Considering the experiments with *E. coli*, it is likely that *Streptomyces* experiments on a clinostat would have similar results as space flight, achieving higher product yields than in similar 1 *g* controls. If so, this would be an excellent "next step" in transferring the knowledge gained from space-based research to terrestrial pharmaceutical production.

Because *Streptomyces* is aerobic, these experiments would likely require a Gas Exchange (GE) membrane in place of one or more septa. Unfortunately, some preliminary clinostat experiments with a GE membrane demonstrated a serious problem that would have to be solved. After approximately one day of growth, the samples often developed bubbles rotating inside the FPA. These bubbles destroy the quiescent fluid environment of the clinostat, making the simulation of "functional weightlessness" invalid. The bubbles, however, are easily removed by pushing a plunger against the membrane. This problem could possibly be solved by placing the clinostat samples (and the controls) in a humid environment to prevent evaporation.

9.3.3 Antibiotics

It is widely accepted that antibiotics are less effective on orbit (reference Table 2-1 and 2-4). However, it is still unknown why this occurs. Perhaps the theory of buoyancy-driven convection could help explain these results. It is plausible that the lack of convection on orbit reduces the interaction between antibiotics and bacteria,

making some antibiotics less effective on orbit. It is also possible that the "cloud" of excreted by-products around bacteria in space alters the surrounding pH, which might reduce the effectiveness of some antibiotics. This could be investigated using ground experiments with horizontal and inclined clinostats as well as hypergravity experiments with a centrifuge.

9.3.4 Crystal Growth

The idea that bacteria are affected by buoyancy-driven convection was derived from a similar phenomenon that affects crystal growth. As a crystal grows it creates a solute depletion zone, which causes small convection currents around the crystal (McCay and McCay, 1994; Pusey and Naumann, 1986; Pusey *et al.*, 1988, 1986). Because these convection currents do not occur on orbit, crystals have larger and more uniform growth in space. This is potentially a very profitable area of space-based research.

It is possible that large crystals could also be grown on the ground using a clinostat. The research in this paper shows that clinorotation and space flight have similar effects on bacterial growth and metabolism. It is, therefore, likely that clinorotation would affect crystal growth in the same manner as space flight. If a growing crystal is fixed in a clinostat near the axis of rotation, a sufficient rotational rate should be able to keep the solute depletion zone near the crystal. This would likely result in a larger and more uniform crystal, without having to go into orbit.

9.3.5 Analyses of Forces at the Cellular Level

Data indicated that bacterial growth creates buoyancy-driven convection. A plume rising from many bacteria was observed (Figure 6-1, page 100). Mathematical analysis showed that the theoretical plume velocity, the Reynolds number, and the boundary layer associated with this flow agreed with observed results. The equation for plume velocity was then applied to convection from a single bacterium with a diameter of 1.2 μm. Unfortunately, this equation has only been validated for plumes as small as 10 μm in diameter (Pusey *et al.,* 1988) and the theoretical plume velocity from a single cell could not be compared with observed data. Future research should try to detect a plume from a single bacterium and determine if a boundary layer exists around the cell. Of particular interest is the region inside the assumed boundary layer. In this region, the standard equations governing diffusion, sedimentation, and buoyancy-driven convection may not be valid. It is therefore important to quantify the interactions between the bacterium, by-products, and nutrients inside the boundary layer.

9.4 CONCLUSIONS

This dissertation proposed a theory to explain the reports of altered bacterial growth and metabolism on orbit. It was hypothesized that inertial acceleration affects bacterial growth by altering the transport phenomena in the cells' external fluid environment. It is believed that this occurs indirectly through changes in the

sedimentation rate acting on the bacteria and buoyancy-driven convection acting on their excreted by-products.

This theory was supported by mathematical analysis and computer simulations. It was also supported by over 20 experiments that were conducted in space, simulated weightlessness, simulated partial gravity, and on a centrifuge. A summary of the major findings includes:

1. A higher concentration of excreted by-products surrounding bacterial cells results in a shorter lag phase.

2. Final cell density is inversely related to the level of acceleration. Acceleration reduces final cell density in two ways. Sedimentation concentrates the cells near the bottom of the culture container, which reduces their nutrient availability. Higher levels of acceleration also increase the buoyant forces that separate by-products from their cells. Both of these gravity-dependent processes reduce the final cell density.

3. The lag phase is affected by acceleration in a predictable non-monotonic manner, with the shortest lag phase occurring between approximately 10 *g* and 100 *g*. There are two gravity-dependent forces that affect the length of the lag phase. Between 1 *g* and 50 *g*, sedimentation is the dominant gravity-dependent force. It clusters bacteria at the bottom of the culture tube, permitting a higher

concentration of by-products around the cells. This reduces the lag phase. As acceleration increases above 50 *g*, buoyant forces increase, which reduce the by-product concentration around the bacteria and increase the length of the lag phase. Eventually, around 400 *g*, there is essentially no difference in the length of the lag phase relative to 1 *g* controls. At hypogravity, both real and simulated, the reduced effectiveness of buoyant forces permits by-products to remain near their cells, which shortens the lag phase relative to 1 *g* control samples.

4. *E. coli* metabolize glucose more efficiently at lower levels of acceleration. Relative to 1 *g* controls, real or simulated hypogravity results in a higher biomass yield, while hypergravity reduces the biomass yield. This is caused by the changes in the buoyant plume velocity that separates important by-products from their cells. At lower levels of acceleration the buoyant forces are reduced, and *E. coli* can metabolize glucose more efficiently.

5. Gravity affects bacterial growth and metabolism indirectly, through changes in the fluid environment surrounding the bacteria. Clinorotation only simulates the "indirect" effects of microgravity. Because clinorotation and space flight have similar effects on bacterial growth and metabolism, we can conclude that gravity only affects bacterial growth and metabolism indirectly.

6. Bacterial growth causes buoyancy-driven convection, and this force can explain the non-linear effects of acceleration on bacterial growth kinetics. A photograph provided evidence of buoyancy-driven convection associated with bacterial growth. Mathematical analysis showed that this is a dominant gravity-dependent force that can explain the observed plume. It also showed that the plume velocity from a single cell is significantly greater than the sedimentation rate of a bacterium. Additional analysis suggested that final cell density is inversely proportional to plume velocity for accelerations greater than about 10 *g*. Also, the length of the lag phase appears to be directly proportional to plume velocity for accelerations greater than about 50 *g*. Therefore, for accelerations above 10 to 50 g, final cell density is a function of $g^{-1/2}$, and the length of the lag phase is a function of $g^{1/2}$.

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[1] Il'in, V. K. and Ilyin, V. K. are different translations of the same Russian name. The spellings used here are consistent with each journal.

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APPENDIX A

FORTRAN COMPUTER CODE

AND RELATED CALCULATIONS

A.1 COMPUTER CODE

The flow-chart displayed in Figure A-1 provides an an overview of the computer program. The computer code is shown on the following pages.

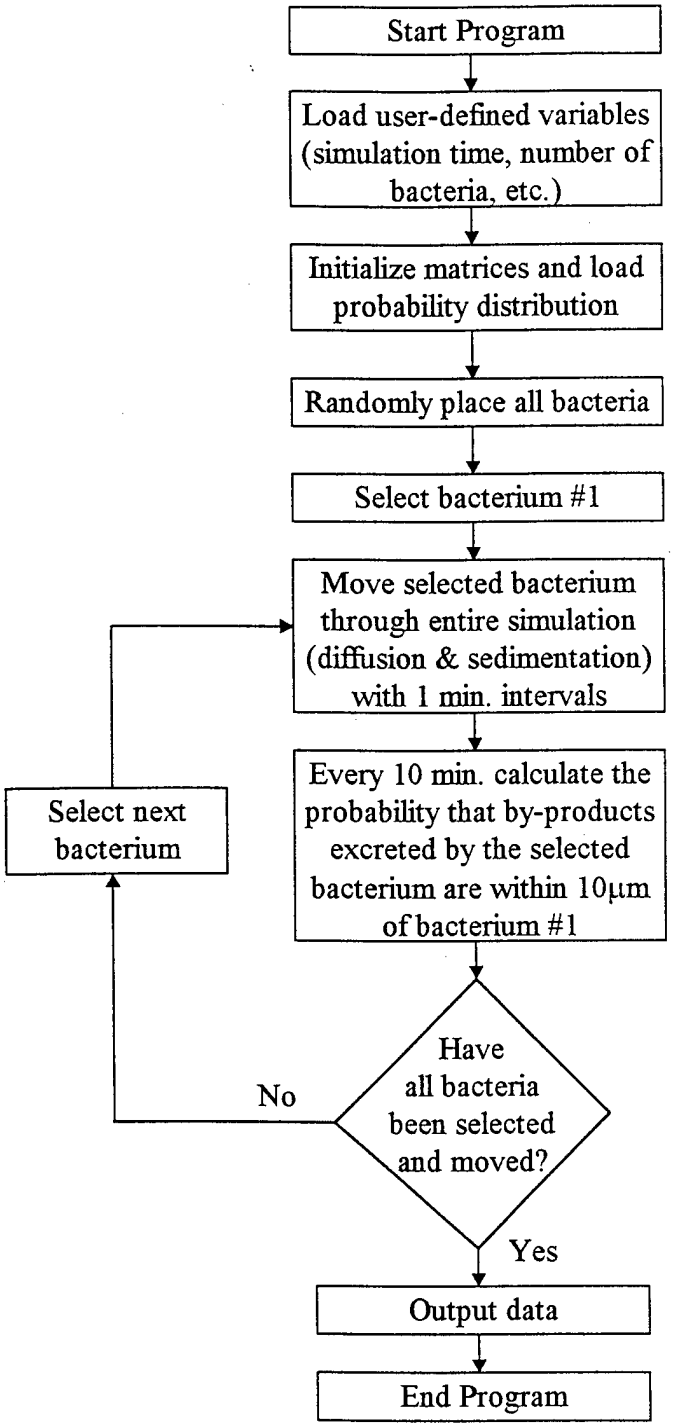

Figure A-1 Simulation Flow-Chart

```
*-------------------------------------------------------------------------------
*
*                              Program Bacteria.for
*
* This program is a simulation of E coli under various gravity conditions in two
* dimensions.  It calculates the probability of one unit of waste within a specified
* range of one specific bacterium.
*
* Author          : Rob Brown
* Last changed    : August, 1998
*
* Subroutines Called:
*   Initialize    : Initializes key parameters
*   First_bact    : Calculates the random position for the first bacteria
*                   (the one that by-product concentration will be
*                   determined from) during the entire simulation and
*                   finds the probability that it's own waste is within
*                   a specified distance at different times.
*   Move          : Moves a bacterium (sedimentation and diffusion)
*   Probability   : Determines the probability waste is within a specified
*                   distance of the first bacterium at a given time
*   Output        : Saves relevant output to data file.
*
* Integer Variables:
*   Bact_Num      : The number of bacteria in the simulation
*   Bottom        : Array that records when each bacterium hits the bottom
*                   of the test tube (up to 50 bacteria) (seconds)
*   Pos           : Array of two bacteria's positions in two dimensions for
*                   every time.  One is for the first bacterium, which is the
*                   one of interest.  The other position is for another bacterium,
*                   which is overwritten for the 2nd, 3rd, 4th, etc. bacteria.
*                   Units are in integers; 1/10 of a um.  This allows better
*                   resolution than 1 um, but still allows the array to be saved
*                   as an integer.
*   Range         : Distance of interest between the first bacterium and
*                   by-products (um).  Generally 10 um
*   Delt          : Time step size (sec)
*   End_t         : Time that the simulation ends (sec)
*   Clock         : Current time (sec)
*   I             : Counter
*   Int_pos       : Array of the initial positions of every bacteria (1/10 um)
```

```
*
*  Real Variables:
*    Norm          : Array of probability distribution for a normal distribution
*    Waste_D       : Diffusion coefficient of by-products (um^2/sec)
*    Gravity       : Number of gs for simulation
*    Bact_D        : Diffusion coefficient of bacteria (um^2/sec)
*    Dis_Sed       : The distance each bacterium sediments in one time step (um)
*    Seed          : Seed for the random number generator
*    RMS           : Root Mean Square diffusion distance (um)
*    Prob          : Array of probability of encountering one unit of waste within
*                    the distance "Range" of the first bacterium for every 10 min
*
* ------------------------------------------------------------------------------------------
      PROGRAM BACTERIA
      IMPLICIT NONE
      INTEGER BACT_NUM, BOTTOM(50), POS(2,2,1801), RANGE, DELT,
     &   END_T, CLOCK, I, INT_POS(50,2)
      REAL NORM(400), WASTE_D, GRAVITY, BACT_D, DIS_SED, SEED,.
     &   PROB(2,360), RMS
      COMMON /ARRAYS/ POS, NORM

*------------------------ Open data files ----------------------------*
      OPEN(UNIT=9, FILE='NORM.DAT',STATUS='OLD', ACCESS =
     &   'SEQUENTIAL')
      OPEN(UNIT=10, FILE='ONE50.DAT',STATUS='UNKNOWN',ACCESS=
     &   'SEQUENTIAL')
      OPEN(UNIT=11, FILE='POS.DAT', STATUS='UNKNOWN',ACCESS=
     &   'SEQUENTIAL')

*---------- Call Subroutines ----------------------------------------*
      GRAVITY = 1
      BACT_NUM = 1
      SEED = .1
      WRITE(*,*) 'GRAVITY ,',GRAVITY,'-G'
      WRITE(*,*) 'BACT_NUM,',BACT_NUM
      CALL INITIALIZE (GRAVITY, WASTE_D, RANGE, DELT, END_T,
     &    BACT_D, DIS_SED, RMS, SEED, BACT_NUM, PROB, BOTTOM,
     &    INT_POS)
      SEED = .1
      CALL FIRST_BACT (WASTE_D, RANGE, DELT, END_T,
     &    DIS_SED, RMS, SEED, PROB, BOTTOM)
      DO I = 2, BACT_NUM
        WRITE(*,*)'BACTERIA # ',I
        POS(2,1,1) = INT_POS(I,1)
        POS(2,2,1) = INT_POS(I,2)
```

```
      CLOCK = 0
      DO WHILE (CLOCK .LT. END_T)
        CLOCK = CLOCK + DELT
        CALL MOVE (DELT, CLOCK, DIS_SED, SEED, I, BOTTOM, RMS)
c-------- Every 10 min check the probability ------------------------
        IF (MOD(CLOCK,600).EQ.0) THEN
          CALL PROBABILITY (CLOCK, DELT, WASTE_D, RANGE, PROB, 2)
        ENDIF
      ENDDO
    ENDDO
    CALL OUTPUT (GRAVITY, PROB, BACT_NUM, BOTTOM)
    CLOSE (9)
    CLOSE (10)
    CLOSE (11)
    STOP
    END
* ---------------------------------------------------------------------------------------------
*
*                    SUBROUTINE INITIALIZE
*
* This subroutine initializes many of the variables used in the program
* Only inputs are Gravity and Seed.
*
* Subroutines Called:
*   Uniform        : Generates a uniform random variable
*
* Integer Variables:
*   Range          : Distance of interest between the first bacterium and
*                    by-products (um).  Generally 10 um
*   Bact_Num       : The number of bacteria in the simulation
*   Bottom         : Array that records when each bacterium hits the bottom
*                    of the test tube (up to 50 bacteria) (seconds)
*   Pos            : Array of two bacteria's positions in two dimensions for
*                    every time.  One is for the first bacterium, which is the
*                    one of interest.  The other position is for another bacterium,
*                    which is overwritten for the 2nd, 3rd, 4th, etc. bacteria.
*                    Units are in integers; 1/10 of a um.  This allows better
*                    resolution than 1 um, but still allows the array to be saved
*                    as an integer
*   Delt           : Time step size (sec)
*   End_t          : Time that the simulation ends (sec)
*   I, J, K        : Counters
*   Int_pos        : Array of the initial positions of every bacteria (1/10 um)
*
* Real Variables:
```

```
*   Norm        : Array of probability distribution for a normal distribution
*   Waste_D     : Diffusion coefficient of by-products (um^2/sec)
*   Gravity     : Number of gs for simulation
*   Bact_D      : Diffusion coefficient of bacteria (um^2/sec)
*   Vel_sed     : Sedimentation velocity (um/sec)
*   Dis_sed     : The distance each bacterium sediments in one time step (um)
*   Seed        : Seed for the random number generator or the random number
*   RMS         : Root Mean Square diffusion distance (um)
*   Den_Bac     : Density of Bacteria (g/cm^3)
*   Den_Med     : Density of Medium (g/cm^3)
*   Bact_a      : Effective stokes radius of bacteria (um)
*   Vis_med     : Viscosity of Medium (g/(cm*sec))
*   Temp        : Temporary variable used when reading in the normal distribution
*   Prob        : Array of probability of encountering one unit of waste within
*                   the distance "Range" of the first bacterium for every 10 min
* ------------------------------------------------------------------------------
      SUBROUTINE INITIALIZE (GRAVITY, WASTE_D, RANGE, DELT,END_T,
     &       BACT_D, DIS_SED, RMS, SEED, BACT_NUM, PROB, BOTTOM,
     &       INT_POS)
      IMPLICIT NONE
      INTEGER RANGE, DELT, END_T, POS(2,2,1801), I, J, K, BACT_NUM,
     &      INT_POS(50,2), BOTTOM(50)
      REAL GRAVITY, WASTE_D, BACT_D, DIS_SED, VEL_SED, NORM(400),
     &       BACT_A, VIS_MED, DEN_BAC, DEN_MED, RMS, TEMP, SEED,
     &       PROB(2,360)

      COMMON /ARRAYS/ POS, NORM

c---- User sets the following variables -----
      BACT_A = .62
      VIS_MED = 0.01
      DEN_BAC = 1.08
      DEN_MED = 1.011
c---- Diffusion coefficient in one dimension
c---- 10 um^2/sec = 10^-7 cm^2/sec
      WASTE_D = 10
      RANGE = 10
      DELT = 60
      END_T = 108000
c----- First convert eff. stokes radius to cm (back to um later)
      BACT_A = BACT_A/10000
      BACT_D = 0.1
      VEL_SED = 2.0/9.0*(DEN_BAC-DEN_MED) *
     &            ((980*GRAVITY)/VIS_MED)*BACT_A**2
      VEL_SED = VEL_SED*10000
```

```
      DIS_SED = VEL_SED*DELT
      RMS = SQRT(2*BACT_D*DELT)
c---  Load Gaussian distribution data (norm.dat)
      DO I = 1, 400
       READ(9,*) TEMP, NORM(I)
      ENDDO
c---- Fill Prob and Bottom with zeros
      DO I = 1,41
       BOTTOM(I) = 0
      ENDDO
      DO I = 1,2
       DO J = 1, 360
        PROB(I,J) = 0.0
       ENDDO
      ENDDO

c---- Initial positions for bacteria (first one is set, rest are random)
c---- First bacterium placed in middle of test tub, 5 mm from the
c---- bottom = 5,000 um = 5,000*10 1/10 um.
      DO I = 1,2
       DO J = 1,2
        DO K = 1, 1801
         POS(I,J,K) = 0
        ENDDO
       ENDDO
      ENDDO
      INT_POS(1,1) = 5000*10
      POS(1,1,1) = 5000*10
      INT_POS(1,2) = 0
      POS(1,2,1) = 0
      DO I = 2, BACT_NUM
       CALL UNIFORM (SEED)
       INT_POS(I,1) = SEED*10000*10
       CALL UNIFORM (SEED)
       INT_POS(I,2) = (SEED*2000 - 1000)*10
      ENDDO
      WRITE(11,*)'Bacteria Initial Positions in mm'
      DO I = 1, BACT_NUM
       WRITE(11,*)I,',',INT_POS(I,2)/10000.0, ', ',
     &          INT_POS(I,1)/10000.0
      ENDDO
      RETURN
      END
```

```
*-------------------------------------------------------------------------------
*
*                    SUBROUTINE FIRST_BACT
*
* This subroutine moves the first bacteria (the one of interest) through
* the entire simulation and keeps track of the probability it is within a
* specified distance of one unit of its own waste, which were released at
* previous times and locations.
*
* Subroutines Called:
*   Move          : Moves bacterium due based on sedimentation and diffusion
*   Probability   : Determines the probability waste is within a specified
*                   distance of the first bacterium at a given time
*
* Integer Variables:
*   Bottom        : Array that records when each bacterium hits the bottom
*                   of the test tube (up to 50 bacteria) (seconds)
*   Pos           : Array of two bacteria's positions in two dimensions for
*                   every time.  One is for the first bacterium, which is the
*                   one of interest.  The other position is for another bacterium,
*                   which is overwritten for the 2nd, 3rd, 4th, etc. bacteria.
*                   Units are in integers; 1/10 of a um.  This allows better
*                   resolution than 1 um, but still allows the array to be saved
*                   as an integer.
*   Range         : Distance of interest between the first bacterium and
*                   by-products (um).  Generally 10 um
*   Delt          : Time step size (sec)
*   End_t         : Time that the simulation ends (sec)
*   Clock         : Current time (sec)
*
* Real Variables:
*   Norm          : Array of probability distribution for a normal distribution
*   Waste_D       : Diffusion coefficient of by-products (um^2/sec)
*   Dis_Sed       : The distance each bacterium sediments in one time step (um)
*   Seed          : Seed for the random number generator
*   RMS           : Root Mean Square diffusion distance (um)
*   Prob          : Array of probability of encountering one unit of waste within
*                   the distance "Range" of the first bacterium for every 10 min
*
*-------------------------------------------------------------------------------
      SUBROUTINE FIRST_BACT (WASTE_D, RANGE, DELT, END_T,
     &    DIS_SED, RMS, SEED, PROB, BOTTOM)
      IMPLICIT NONE
      INTEGER BOTTOM(50), POS(2,2,1801), RANGE, DELT, END_T, CLOCK
      REAL NORM(400), SEED, WASTE_D, DIS_SED, RMS,
```

```
     &  PROB(2,360)
      COMMON /ARRAYS/ POS, NORM
      CLOCK = 0
      DO WHILE (CLOCK .LT. END_T)
       CLOCK = CLOCK + DELT
       CALL MOVE (DELT, CLOCK, DIS_SED, SEED, 1, BOTTOM, RMS)
c------ Every 10 min check the probability of waste within "Range" of bacterium
       IF (MOD(CLOCK,600).EQ.0) THEN
        CALL PROBABILITY (CLOCK, DELT, WASTE_D, RANGE, PROB, 1)
       ENDIF
      ENDDO
      RETURN
      END

* ------------------------------------------------------------------------------------
*
*                    SUBROUTINE MOVE
*
* This subroutine calls rnum to generate a random variable and moves a
* bacterium a distance RMS in both dimensions and a distance dis_sed downward.
*
* Subroutines Called:
*   Normal        : Generates two normal random numbers
*
* Integer Variables:
*   Bottom        : Array that records when each bacterium hits the bottom
*                   of the test tube (up to 50 bacteria) (seconds)
*   Pos           : Array of two bacteria's positions in two dimensions for
*                   every time.  One is for the first bacterium, which is the
*                   one of interest.  The other position is for another bacterium,
*                   which is overwritten for the 2nd, 3rd, 4th, etc. bacteria.
*                   Units are in integers; 1/10 of a um.  This allows better
*                   resolution than 1 um, but still allows the array to be saved
*                   as an integer.
*   Delt          : Time step size (sec)
*   Clock         : Current time (sec)
*   Bact_Num      : The number of bacteria in the simulation
*   Num           : Num=1 specifies the first bacterium; Num=2 specifies another
*                   bacterium (2nd, 3rd, etc.) whose position is being generated
*   Current       : Current time slot (3rd place) in Pos Array = Clock/Delt + 1
*
* Real Variables:
*   Norm          : Array of probability distribution for a normal distribution
*   Waste_D       : Diffusion coefficient of by-products (um^2/sec)
*   Dis_Sed       : The distance each bacterium sediments in one time step (um)
```

```
*   Seed            : Seed for the random number generator
*   RMS             : Root Mean Square diffusion distance (um)
*   Norm1, Norm2: Two normal random numbers used to move bacterium in two
*                   dimensions due to diffusion.
* ------------------------------------------------------------------------------------------
      SUBROUTINE MOVE (DELT, CLOCK, DIS_SED, SEED,BACT_NUM,
     &     BOTTOM, RMS)
      IMPLICIT NONE
      INTEGER CLOCK, DELT, BACT_NUM, BOTTOM(50), POS(2,2,1801),
     &    NUM, CURRENT
      REAL DIS_SED, SEED, RMS, NORM(400), NORM1, NORM2
      COMMON /ARRAYS/ POS, NORM
      CURRENT = CLOCK/DELT+1
      NUM = 2
      IF (BACT_NUM.EQ.1) NUM = 1
c---- This algorithm generates two random numbers
c---- one is used for each dimension
      CALL NORMAL (SEED, RMS, NORM1, NORM2)
c---- Add 10 times (accounts for .1 um) the diffusion - sed distance
      POS(NUM,1,CURRENT) = POS(NUM,1,CURRENT-1) +
     &     (NORM1-DIS_SED)*10
      POS(NUM,2,CURRENT) = POS(NUM,2,CURRENT-1) + NORM2*10
c---- See if the bacteria has hit bottom (pos<0) --------------------
      IF (POS(NUM,1,CURRENT).LT.0.0) THEN
        POS(NUM,1,CURRENT) = ABS(POS(NUM,1,CURRENT))
        IF (BOTTOM(BACT_NUM).EQ.0) BOTTOM(BACT_NUM)=CLOCK
      ENDIF
      RETURN
      END
```

```
*-------------------------------------------------------------------------------
*
*                    SUBROUTINE PROBABILITY
*
* This subroutine calculates the probability of encountering waste within a
* given distance, and a given time, and prior locations.
*
* Subroutines Called:
*   Inter          : Interpolates between two points in Norm (a normal distrib. table)
*
* Integer Variables:
*   I, J           : Counters
*   K              : K=1 finds probability of waste between to points.
*                    K=2 used to find probability of waste after it has "reflected" off
*                    the bottom of the test tube (i.e. waste that accumulates on the
*                    bottom of the test tube, and has started to diffuse upward).
*   Pos            : Array of two bacteria's positions in two dimensions for
*                    every time. One is for the first bacterium, which is the
*                    one of interest. The other position is for another bacterium,
*                    which is overwritten for the 2nd, 3rd, 4th, etc. bacteria.
*                    Units are in integers; 1/10 of a um. This allows better resolution
*                    than 1 um, but still allows the array to be saved as an integer.
*   Delt           : Time step size (sec)
*   Bact_Num       : The number of bacteria in the simulation
*   Num            : Num=1 specifies the first bacterium; Num=2 specifies another
*                    bacterium (2nd, 3rd, etc.) whose position is being generated
*   Range          : Specified distance in either dimension around the first bacterium
*                    for calculating the probability of waste near first bacterium
*   Current        : Current time slot (3rd place) in Pos Array = Clock/Delt + 1
*   Prob_cnt       : Current time slot (1st place) in Prob Array. Equal to
*                    Clock/600+1 (every 10 min)
*
* Real Variables:
*   Norm           : Array of probability distribution for a normal distribution
*   Waste_D        : Diffusion coefficient of by-products (um^2/sec)
*   Std            : Standard deviation of distribution = sqrt(2Dt). Equivalent
*                    to the expected diffusion distance
*   Elapsed_T      : Elapsed time since waste was released (sec)
*   X,Y            : The vertical and horizontal separation respectively (um)
*   Z_low,Z_high   : Specifies the range under the z distribution.
*   Low, High      : Probability Z<Z_low and Z<Z_high respectively
*   Two_waste_D: 2*Waste_D
*   Prob_X,Prob_Y: Probability of waste within specified Range in the vertical
*                    and horizontal directions respectively
*-------------------------------------------------------------------------------
```

```
      SUBROUTINE PROBABILITY (CLOCK, DELT, WASTE_D, RANGE, PROB,
     &     NUM)
      IMPLICIT NONE
      INTEGER CLOCK, DELT, I, J, K, RANGE, CURRENT, NUM,
     &    POS(2,2,1801), PROB_CNT
      REAL WASTE_D, NORM(400), STD, ELAPSED_T, X, Y, Z_LOW,
     &   Z_HIGH, TWO_WASTE_D, LOW, HIGH, PROB(2,360), PROB_X,
     &   PROB_Y
      COMMON /ARRAYS/ POS, NORM
      TWO_WASTE_D = 2*WASTE_D
      CURRENT = CLOCK/DELT + 1
      PROB_CNT = CLOCK/600 +1
      DO J = 1, CURRENT - 1
       ELAPSED_T = CLOCK - ((J-1) * DELT)
       STD = SQRT(TWO_WASTE_D * ELAPSED_T)
c------ Find the probability twice.  When k=2, it is for the "reflected" waste
c------ that "hits" bottom and then diffuses upward (vertical direction,
c------ x is treated as a negative value).
c------ Divide by 10 to convert the integer position difference in .1 um into um
       Y = (POS(1,2,CURRENT) - POS(NUM,2,J))/10.0
c------ Find the area under a normal curve between z_low and z_high
       Z_LOW  = (Y-RANGE)/STD
       Z_HIGH = (Y+RANGE)/STD
       CALL INTER (Z_LOW, LOW)
       CALL INTER (Z_HIGH, HIGH)
       PROB_Y = HIGH - LOW
       DO K = 1, 2
        IF (K.EQ.1) X =  (POS(1,1,CURRENT) - POS(NUM,1,J))/10.0
        IF (K.EQ.2) X = -(POS(1,1,CURRENT) + POS(NUM,1,J))/10.0
c-------- Find the area under a normal curve between z_low and z_high
        Z_LOW  = (X-RANGE)/STD
        Z_HIGH = (X+RANGE)/STD
        CALL INTER (Z_LOW, LOW)
        CALL INTER (Z_HIGH, HIGH)
        PROB_X = HIGH - LOW
        PROB(1, PROB_CNT) = CLOCK
        PROB(2, PROB_CNT) = PROB(2,PROB_CNT) + PROB_X*PROB_Y
       ENDDO
      ENDDO
      RETURN
      END
```

```
*-------------------------------------------------------------------------------
*
*                    SUBROUTINE INTER
*
* Interpolates between points to find Phi(z)-.5  given a value for z.
*
*
* Real Variables:
*   Norm          : Array of probability distribution for a normal distribution
*   Z             : Value of z for the normal distribution (number of standard
*                   deviations from the norm)
*   Phi           : Area between Norm and Z
*   Before        : The number just prior to Z in the normal distribution table.
*                   This is used to interpolate between two points in the table.
*-------------------------------------------------------------------------------
      SUBROUTINE INTER (Z, PHI)
      IMPLICIT NONE
      INTEGER POS(2,2,1801)
      REAL NORM(400), Z, PHI, BEFORE
      COMMON /ARRAYS/ POS, NORM
      IF (ABS(Z).GE.3.99) THEN
        PHI = .49997
      ELSE
        BEFORE = INT(ABS(Z*100))+1
        PHI =  (NORM(BEFORE) +
     &    (ABS(Z)-(BEFORE-1)*.01) * (NORM(BEFORE+1)-NORM(BEFORE))/.01)
      ENDIF
      IF (Z.LT.0) PHI = -1*PHI
      RETURN
      END
```

```
* ------------------------------------------------------------------------------
*
*                    SUBROUTINE UNIFORM
*
* Generates a uniform random variable [0,1)
* Reference  Park et al., 1988
*
* Real Variable:
*   Seed           : Random number between 0 and 1 (input and output)
* ------------------------------------------------------------------------------
      SUBROUTINE UNIFORM (SEED)
      IMPLICIT NONE
      REAL SEED
      SEED = MOD(2147483647, 16807*seed)/(16807*seed)
      RETURN
      END

* ------------------------------------------------------------------------------
*
*                    SUBROUTINE NORMAL
*
* Generates two normal random variables
*
* Real Variables:
*   Seed           : Random number between 0 and 1 (can be zero but not 1)
*   Std            : Standard Deviation of Distribution
*   X1, X2         : Random numbers output by subroutine
*   V1, V2, LN     : Used to calculate X1 and X2
* ------------------------------------------------------------------------------
      SUBROUTINE NORMAL(SEED, STD,       X1, X2)
      IMPLICIT NONE
      REAL SEED, STD, X1, X2, S, V1, V2, LN
      S = 2
      DO WHILE (S.GT.1)
        CALL UNIFORM (SEED)
        V1 = 2*SEED-1
        CALL UNIFORM (SEED)
        V2 = 2*SEED-1
        S = V1**2 + V2**2
      ENDDO
      LN = STD * SQRT(-2*LOG(S)/S)
      X1 = V1*LN
      X2 = V2*LN
      RETURN
      END
```

```
*-------------------------------------------------------------------------------
*
*                    SUBROUTINE OUTPUT
*
* Outputs probability data and the times each bacterium reached the bottom.
*
* Integer Variables:
*   I              : Counter
*   Bact_Num       : The number of bacteria in the simulation
*   Bottom         : Array of the times each bacterium reached the bottom of
*                    the test tube (sec)
*
* Real Variables:
*   Gravity        : Number of gs for simulation
*   Prob           : Array of probability of encountering one unit of waste within
*                    the distance "Range" of the first bacterium for every 10 min.
*
*-------------------------------------------------------------------------------
      SUBROUTINE OUTPUT (GRAVITY, PROB, BACT_NUM, BOTTOM)
      IMPLICIT NONE
      INTEGER  I, BACT_NUM, BOTTOM(50)
      REAL PROB(2,360), GRAVITY
      WRITE(10,*) 'Gravity ,',GRAVITY
      WRITE(10,*) 'Hours, Prob'
      DO I = 1, 360
       WRITE(10,40) PROB(1,I)/3600.0, PROB(2,I)
      ENDDO
      DO I = 1, BACT_NUM
       WRITE(10,*)'BACT #',I,' hit bottom at,',BOTTOM(I)/3600.0
      ENDDO

   40  FORMAT(F8.5,','F9.5)
      RETURN
      END
```

A.2 CALCULATIONS FOR *E. coli*

This section shows some of the most common calculations used in this dissertation. This includes many of the parameters used in the computer simulation. *E. coli's* size, surface area, diffusion, and sedimentation rate were calculated using the following parameters (Klaus *et al.*, 1997).

E. coli	
Volume	1×10^{-12} cm^3
Mass	1×10^{-12} g
Density	1.08 g/cm^3
Medium	
Viscosity	3.6 kg/(hr·min)
Density	1.011 g/cm^3

A.2.1 Effective Radius and Surface Area

E. coli's effective Stokes' radius was calculated using the volume given above. This was then used to estimate the average surface area, assuming spherical bacteria.

$$\text{Radius} = a = \left(\frac{3V}{4\pi}\right)^{\frac{1}{3}} = \left(\frac{3\left(1\times10^{-12}\ \text{cm}^3\right)}{4\pi}\right)^{\frac{1}{3}} = 6.2\times10^{-5}\ \text{cm} = 0.62\ \mu\text{m} \qquad \text{(A-1)}$$

$$\text{Surface Area} = 4\pi r^2 = 5\times10^{-8}\ \text{cm}^2 = 5\ \mu\text{m}^2 \qquad \text{(A-2)}$$

A.2.2 Diffusion Coefficient

The diffusion coefficient for *E. coli* was found from the effective Stokes' radius using Equation A-3.

$$D = \frac{k_B T}{6\pi\mu a} = 3\times10^{-9}\ \frac{\text{cm}^2}{\text{sec}} = 0.3\ \frac{\mu\text{m}^2}{\text{sec}} \qquad \text{(A-3)}$$

where k_B = Boltzmann's constant = $1.38\times10^{-23}\ \frac{\text{J}}{\text{molecule}\cdot^\circ\text{K}}$

$$= 1.38\times10^{-16}\ \frac{\text{g}\cdot\text{cm}^2}{\text{sec}^2\cdot\text{molecule}\cdot^\circ\text{K}}$$

T = Temperature in °K = 294 °K

μ = Viscosity of the medium = 3.6 kg/(hr·min) = 0.01 g/(cm·sec)

a = Effective Stokes' radius = 6.2×10^{-5} cm

This coefficient was used to calculate the expected distance *E. coli* diffuses in a given time, using the equation

$$\langle x \rangle \doteq \sqrt{2Dt}\ . \qquad \text{(A- 4)}$$

A bacterium's movement due to diffusion is a normal probability distribution with a standard deviation equal to $\langle x \rangle$ in each dimension (Einstein, 1956). For example, using the coefficient calculated above, the expected diffusion distance of *E. coli* in one second is 0.8 μm in each dimension. Of course, this is just an expected diffusion distance. The actual distance is a random number, with a standard deviation of 0.8 μm.

A.2.3 Sedimentation Rate

The sedimentation rate of *E. coli* in 1 *g* is calculated using the Navier-Stokes' equation, shown in Equation A-5.

$$V_{Sed} = \frac{2}{9}\left(\partial_{Cell} - \partial_{Medium}\right)\left(\frac{g}{\mu}\right)a^3$$

$$= \frac{2}{9}\left(1.08\frac{g}{cm^3} - 1.011\frac{g}{cm^3}\right)\left(\frac{980\,cm^2/sec}{0.01\,g/cm\cdot sec}\right)\left(6.2\times10^{-5}\,cm\right)^3 \qquad \text{(A-5)}$$

$$= 6\times10^{-6}\frac{cm}{sec} = 0.06\frac{\mu m}{sec} = 3.6\frac{\mu m}{min} = 0.022\frac{cm}{hr}$$

The distance an *E. coli* bacterium sediments in a given time is easily found from $V_{sed} \times t$. It is interesting to compare this displacement due to sedimentation, with the random motion due to diffusion. For short time intervals of less than a minute, the expected diffusion distance of a bacterium is much greater than the displacement due to sedimentation. After 166 seconds, however, sedimentation is a more dominant force acting on a bacterium.

Based upon the experimental data presented in Chapter 5, *E. coli* reach the stationary phase after approximately 60 hours in an FPA at 1 *g*. In this time, they have sedimented 0.022×60=1.3 cm. A standard 4 ml sample in an FPA is approximately 4 cm in height. Therefore, approximately one third of the bacteria are theoretically at the bottom of an FPA by the end of the exponential phase.

However, at accelerations above 1 g the bacteria quickly reach the bottom of their test tube. For example at 50 g, it takes approximately 3.6 hours for all the bacteria to theoretically reach the bottom of their culture tube.

$$\frac{\text{Height of Tube}}{\left(\text{Sedimentation Rate per } g\right)\left(50\ g\right)} = t$$

$$\frac{4\text{ cm}}{\left(0.022\ {}^{\text{cm}}/_{\text{hr}}\right)50} = 3.6\text{ hours}$$

A.2.4 Intercellular Separation

When *E. coli* are inoculated with a concentration of 10^6 cells/ml, each cell occupies an average volume of 10^{-6} ml. Assuming this is a spherical volume, its radius is

$$r = \left(\frac{3\left(10^{-6}\text{cm}^3\right)}{4\pi}\right)^{\frac{1}{3}} = 0.006\text{ cm} = 60\ \mu\text{m}. \qquad \text{(A-6)}$$

Therefore, there is initially a separation of approximately 120 μm between *E. coli* bacteria. When the cells reach the stationary phase, with approximately 10^9 cells/ml, this intercellular distance is only 10 μm.

Brown, Robert Bayne (Ph.D., Aerospace Engineering Sciences)

Effects of Space Flight, Clinorotation, and Centrifugation on the Growth and Metabolism of *Escherichia coli*

Thesis directed by Dr. Paul W. Todd and Dr. David M. Klaus

Previous experiments have shown that space flight stimulates bacterial growth and metabolism. An explanation for these results is proposed, which may eventually lead to improved terrestrial pharmaceutical production efficiency. It is hypothesized that inertial acceleration affects bacterial growth and metabolism by altering the transport phenomena in the cells' external fluid environment. It is believed that this occurs indirectly through changes in the sedimentation rate acting on the bacteria and buoyancy-driven convection acting on their excreted by-products.

Experiments over a broad range of accelerations consistently supported this theory. Experiments at 1 *g* indicated that higher concentrations of excreted by-products surrounding bacterial cells result in a shorter lag phase. Nineteen additional experiments simulated 0 *g* and 0.5 *g* using a clinostat, and achieved 50 *g*, 180 *g*, and 400 *g* using a centrifuge. These experiments showed that final cell density is inversely related to the level of acceleration. The experiments also consistently showed that acceleration affects the length of the lag phase in a non-monotonic, yet predictable, manner. Additional data indicated that *E. coli* metabolize glucose less efficiently at hypergravity, and more efficiently at hypogravity. A space-flight experiment was also performed. Samples on orbit had a statistically significant higher final cell density and more efficient metabolism than did ground controls. These results, which were similar

to simulations of 0 g using a clinostat, support the theory that gravity only affects bacterial growth and metabolism indirectly, through changes in the bacteria's fluid environment.

Evidence of buoyancy-driven convection associated with bacterial growth was also obtained by photographing a plume rising from metabolizing bacteria. These plumes were analyzed mathematically. Additional analysis, using a computer simulation, investigated the effects of diffusion and sedimentation. All of the analyses agreed with observed results and supported the proposed theory.

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